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(57) Abstract

The invention describes a previously unknown gene, termed *5'OT–EST*, which is responsible for inducing an obesity and/or infertility phenotype in transgenic animals, and transgenic animals comprising mutants of *5'OT–EST* which are useful for assaying compounds for the treatment of obesity and/or infertility.

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Gene

The present invention relates to a gene which is involved in the control of obesity and fertility. In particular, the gene disclosed herein is involved in late-onset obesity in males, which is coupled with infertility. Moreover, the invention relates to animal models for late-onset obesity.

Obesity, which differs from being overweight by being characterised by an increase in the proportion of body fat present as opposed to a mere increase in body weight, is one of the major contributors to chronic disease development. Mortality in overweight males (5-15% overweight) increases to 125%, but rises up to 500% in obese males. Laboratory and epidemiological studies have also shown that mortality amongst obese males aged between 25 and 34 can increase up to twelve times. This increase in mortality is caused by the multitude of health risks associated with obesity, including cardiovascular disease, hypertension, diabetes, sleep apnoea (the abnormal ceasing of breathing during sleep), hernias, flat feet, arthritis, osteoarthritis, some cancers, varicose veins, gout, respiratory problems, gall bladder disease and liver disease. The more serious complaints include:

- Cardiovascular Disease – Obesity is an important factor in cardiovascular disease in both increasing blood cholesterol and blood pressure, and has been shown to increase the risk of disease by up to three times. Obesity also increases the work of the heart – cardiac volume, stoke volume and blood volume must all increase to cope with the increased weight. The detrimental effects of obesity on cardiovascular disease are reversible with weight loss.

- Diabetes – Excess weight also increases the chance of acquiring diabetes mellitus by threefold and increases the risk of dying from diabetes by up to eight times. The mechanism by which an increase in body fat increases the risk of diabetes is largely unknown. However, it is postulated that a slight increase in circulating serum glucose or L-leucine may increase the basal levels of insulin. This rise in insulin is then associated with resistance to insulin, caused both by decreased intracellular effects of insulin and a reduction in insulin receptors in the cell. Obesity has also been proven to

alter pancreatic function and in susceptible individuals this may lead to the development of diabetes mellitus .

- Cancer - cancer has also shown a significant association with obesity, but the mechanisms are not understood. One proposed explanation is that obesity alters hormone levels and this could influence cancer development. In males, obesity is associated with a greater risk of developing prostate and colorectal cancers.

- Gall bladder Disease – obese males show a four times increase in the risk of developing gall bladder disease.

- Endocrine Function – this is also modified by elevated fat levels. The Beta cells in the islets of Langehans are enlarged in obese people and glucose intolerance is also frequently inhibited.

- Reproductive System – obesity in males also impairs the functioning of the reproductive system.

- Growth Hormone – obesity impairs the release of growth hormone from the pituitary gland. This problem is of particular importance in obese children whose growth may be impaired. It is fully reversible if weight is lost.

Numerous genes, gene products and their receptors have been characterised in rodent models of obesity which bear mutations associated with different forms of obesity (Bray & York, 1979; Comuzzie & Allison, 1998). Most such spontaneous mutations are recessive, and include mutations affecting leptin and its receptors in such models as ob/ob and db/db mice, Zucker fa/fa rats, Koletsky (f) rats, OLETF rats, corpulent (cp) rats and their substrains or derivatives (Zhang *et al.*, 1994; Tartaglia *et al.*, 1995; Iida *et al.*, 1996; Takaya *et al.*, 1996; Chen *et al.*, 1996; Jamal *et al.* 1997; Kahle *et al.*, 1997; Lee *et al.*, 1997; Moon & Friedman, 1997; Takiguchi *et al.*, 1998). These phenotypes are thought to result from a disruption in leptin or its receptors or in CCK-A receptors, and affect the control of food intake or energy expenditure or metabolism, and disrupt the gonadotrophic

axis in females.

There are numerous other candidate genes putatively involved in obesity, some of which have been recently been summarised by Comuzzie & Allison (1998; Table 1). These include tubby (tub), agouti , Nhl2, MCH, CRH , hypocretins or orexins, CART peptides, melanocortin-4 ligands, uncoupling proteins (UCP1-3), carboxypeptidase E, NPY, their related transcripts or homologues or their receptors (Coleman *et al.*, 1990; Miller *et al.*, 1993; Good *et al.*, 1997; Klebig *et al.*, 1995; Naggert *et al.*, 1995; Ollman *et al.*, 1995; Kleyn *et al.*, 1996; Richard, 1996; Qu *et al.*, 1996; Fan *et al.*, 1997; Huszar *et al.*, 1997; North *et al.*, 1997; Ohki-Hamazaki *et al.*, 1997; Graham *et al.*, 1997; Boss *et al.*, 1997; Vidal-Puig *et al.*, 1997; Millet *et al.*, 1997; Cool *et al.*, 1997; Kristensen *et al.*, 1998; De Lecea *et al.*, 1998; Sakurai *et al.*, 1998). These models exhibit some degree of sexual dimorphism, a slight delay in onset of obesity or a dominant pattern of inheritance, though none show all of these in combination. It is generally believed that obesity is due to the complex interaction of a number of different factors.

The study of obesity and its effects on health requires suitable animal models which can faithfully replicate the condition as seen in humans. None of the available models combines all of the symptoms of obesity. In particular, the symptoms of male pattern obesity, which include late onset, sterility and a concentration of fat around the abdomen, are not displayed by currently available models. There is therefore a need for an improved model for obesity, which displays more of the characteristics of obesity observed in human patients.

Transgenesis is a well established technique for the introduction of DNA sequences into the mammalian genome, and has been used to insert endocrine genes in several species, predominantly in mice (Palmiter *et al.*, 1982; Bucchini *et al.*, 1986; McGrane *et al.*, 1988; Ho *et al.*, 1995), but also in other species (Hammer *et al.*, 1985; Pursel *et al.*, 1989), including rats (Mullins *et al.*, 1990, Zeng *et al.*, 1994, Chareau *et al.*, 1996; Flavell *et al.*, 1996). The methods are well described (Hogan *et al.*, 1986, Chareau *et al.*, 1996) and usually involve the microinjection of cloned DNA fragments into the male pro-nuclei of eggs isolated from superovulated females. Such eggs are transferred into the oviduct of

pseudopregnant females (obtained by mating with vasectomized males) and carried to term. DNA extracted from tail clippings obtained from the progeny may be examined for the presence of specific transgene DNA. Depending on the integrity and stability of the DNA sequence, the number of integration sites and their location in the host genome, transgenes may become stably integrated in the host genome and transmitted to subsequent progeny.

If promoter and enhancer sequences are present in the transgene, the transgene may show high levels of expression in the host animals and the products may induce an endocrine phenotype that would be expected from the hormone product. For example, overexpression of human growth hormone (hGH) using a variety of heterologous non-specific promoters induces variable degrees of growth stimulation in transgenic animals (Palmiter *et al.*, 1982, 1983; Morello *et al.*, 1986; Pursel *et al.*, 1989; Shanahan *et al.*, 1989; Stewart *et al.*, 1992; Short *et al.*, 1992). However, transgene expression levels often differ between different transgenic lines made with the same insert, and the tissue specificity may vary, being highly dependent on the size of the DNA insert, the number of copies of the insert, its integrity and its integration site(s) in host DNA (Lacy *et al.*, 1983; Al-Shawi *et al.*, 1990; Huber *et al.*, 1994). Unexpected phenotypes may result, either as pathological consequences of inappropriate amounts of transgene product or its production in ectopic sites, and examples of this for hGH transgenes include glomerulosclerosis or female infertility in mice or rats (Bartke *et al.*, 1988; Brem *et al.*, 1989; Quaife *et al.*, 1989; Ninomiya *et al.*, 1994). Intentionally directed expression of a transgene to an ectopic site may also have a significant influence on the nature of the phenotype produced (Ornitz *et al.*, 1985; Baker *et al.*, 1992). This is well exemplified using hGH transgenes, since instead of an overgrowth phenotype, hGH can produce an opposite, dwarf phenotype in transgenic mice or rats when driven by a promoter that targets it to the central nervous system to induce negative feedback effects on the endogenous GH system (Hollingshead *et al.*, 1989, Banerjee *et al.*, 1994; Szabo *et al.*, 1995, Flavell *et al.*, 1996).

Other examples of endocrine transgenes include those targeting the genes for oxytocin (OT) and vasopressin (AVP) (Russo *et al.*, 1988; Habener *et al.*, 1989; Grant *et al.*,

1993a,b; Ang *et al.*, 1991, 1994; Murphy & Ho, 1995). These genes are expressed mainly in magnocellular neurones of the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus (Vandesande *et al.*, 1975; Young, 1992; Gainer & Wray, 1994). The expression of these hormonal peptides appears to be mutually exclusive, coexpression in the same neurone occurring only rarely (Kiyama *et al.*, 1990). A construct consisting of sequences 0.6 kb 5', 1.8 kb 3' and the entire structural gene of bovine OT directed expression to the oxytocinergic cells of the SON and the PVN, but also to the lung and Sertoli cells of the testis in transgenic mice (Ho *et al.*, 1995). The hypothalamic expression was also physiologically regulated with an increase in the abundance of the transgene transcript occurring during dehydration. The Sertoli cells are a site of peripheral expression of the endogenous OT gene in cattle but not in mice or rats. In these transgenic mice, the testicular transcripts are translated and processed (Ang *et al.*, 1994), suggesting that this construct contained regulatory elements capable of recapitulate the bovine expression pattern of OT in the mouse testis (Ang *et al.* 1991). Foo *et al.* (1994) have identified a testis-specific promoter in the rat AVP gene.

The AVP and OT genes are highly homologous in structure, and are transcribed in opposite orientations from positions closely linked in the genome within a single locus (Sausville *et al.*, 1985; Young, 1992). It is therefore possible that elements in the flanking sequence of the OT gene normally interact with those present in the nearby homologous AVP gene to regulate their mutually exclusive expression (Young *et al.*, 1990; Young, 1992). To test this theory, mice were generated bearing 1.25 kb of 5', 0.2 kb of 3' and the structural gene for bovine AVP fused, in same the same orientation as the endogenous genes, to the bovine OT transgene already described to show hypothalamic expression (Ho *et al.*, 1995). The resulting mice expressed the bovine OT transgene in the testis and lung, but lacked hypothalamic expression of this transgene and did not express the bovine AVP transgene. A further bovine OT transgene, including 3 kb of 5' sequence, the structural gene and 2.5 kb of downstream sequence was used in an attempt to overcome this repression, but no animals were generated, and it was argued that the region between 0.6 kb and 3 kb 5' of the bovine OT gene conferred a toxic effect in embryonic development which is usually repressed (Ho *et al.*, 1995). Transgenic rats have also been generated bearing fragments of the rat AVP gene with reporter genes inserted

into the third exon of the AVP gene. Transgenes containing 1.5 kb and 3kb of 5', 0.2kb of 3' and the rat AVP gene with a ß-galactosidase reporter gene in the third exon also conferred expression to the testis. This was attributed to the presence of a cryptic testicular promoter within the reporter gene (Zeng *et al.*, 1994a). Clearly however, the use of small fragments of DNA containing OT or AVP sequences gives rise to unpredictable patterns of transgene expression.

One theory is that this variation may be overcome if sufficiently large DNA constructs are used, containing regions of DNA known as locus control regions (LCRs) that can direct tissue specific, position independent, copy number dependent, physiologically appropriate, expression of the transgene in the host (Grosveld *et al.*, 1987; Bonifer *et al.*, 1990; Huber *et al.*, 1994; Fujiwara *et al.*, 1997). Again this may be exemplified with hGH transgenes. An LCR region for the hGH gene has been defined by Jones *et al.* (1995). When a cosmid containing this sequence was used to generate several lines of transgenic mice, hGH was expressed in the pituitary gland in an appropriately regulated fashion, and the mice showed no overgrowth phenotype or other pathological consequences of overproduction of hGH.

Summary of the invention

A line of transgenic rats has been generated using a cosmid of rat DNA containing the genes for oxytocin (OT) and vasopressin (AVP), into which reporter genes were inserted, namely hGH (Roskam *et al.*, 1979) in the AVP gene and bovine OT mostly replacing the rat OT gene. To attempt to include LCR regions for this gene locus in our transgene constructs, larger DNA fragments containing both OT and AVP genes and larger amounts of flanking sequences were used, which were isolated from a rat cosmid library. One line of such rats, bearing at least 4 copies of this cosmid as a concatamer integrant, exhibits an unexpected and novel late onset obesity and infertility dominant phenotype that would not be predicted from the known DNA sequences present in this cosmid. This phenotype is clearly distinguishable from other obesity/infertility syndromes so far described.

Analysis of the cosmid sequences used in the transgene constructs reveals the presence of a previously unknown gene, which is responsible for the observed obesity phenotype.

Accordingly, in the first aspect of the present invention there is provided a 5'OT-EST polypeptide having a sequence selected from the group comprising the sequences set forth in any one of SEQ. ID. Nos. 2, 4 or 6, and sequences substantially homologous to any one of the polypeptides set forth in SEQ. ID. Nos. 2, 4 or 6.

In a second aspect, the invention provides a mutant of a 5'OT-EST polypeptide according to the first aspect of the invention which is capable, *in vivo*, of modulating the obesity of an animal expressing it.

In a third aspect, the present invention provides a nucleic acid encoding a 5'OT-EST polypeptide or mutant 5'OT-EST polypeptide according to the first aspect of the invention. Advantageously, the nucleic acid has a sequence selected from the group consisting of any one of SEQ. ID. Nos. 1, 3, 5 or 7; sequences which are hybridisable under stringent conditions with an oligonucleotide comprising 20 contiguous bases from any one of SEQ. ID. Nos. 1, 3, 5 or 7; sequences substantially homologous to any one of SEQ. ID. Nos. 1, 3, 5 or 7; and sequences complementary thereto. Stringent hybridisation conditions are preferably as defined below.

In a fourth aspect, the invention provides diagnostic reagents for the detection of mutations, polymorphisms or other changes in *5'OT-EST* which may predispose an individual to obesity. For example, the invention provides probes useful for amplifying *5'OT-EST* nucleic acids.

In a fifth aspect, the invention provides a transgenic non-human animal expressing, as a result of transgene expression, a 5'OT-EST polypeptide or mutant 5'OT-EST polypeptide according to the invention. Transgenic animals according to this aspect of the invention are models for obesity in humans, and may be used for research into therapies and treatments which may be used to alleviate obesity.

Brief Description of the Drawings

Figure 1 shows a partial restriction map of the rat AVP/OT locus, from cosmid cVO1, cVO2 and cVO3. Restriction sites for the enzymes listed are shown as a vertical marks. The known sequence of the rat AVP and OT genes is indicated by single dashed lines, and the sequence determined and disclosed herein of the rat *5'OT-EST* gene is indicated by the double dashed line. Scale is approximate.

Figure 2 is a diagrammatic representation of the subcloning steps leading to the insertion of the hGH reporter gene into the 5' untranslated region of the rat vasopressin gene. The final subclone shows the Cla 1 to Xho 1 fragment which was inserted into the construct used to make transgenic lines.

Figure 3 is a diagrammatic representation of the subcloning steps required for the production of the rat-bovine hybrid gene which was inserted into the final construct. For simplicity, this is shown in reversed orientation compared to its orientation in the construct.

Figure 4 shows the extent of the rat AVP/OT locus present in the cosmid cVO1, 2 and 3. These clones span a total of 44kb, including 8kb 5' of rAVP and 24kb 5' of rOT. The structure of the final cosmid construct CVO14 is illustrated and some restriction sites indicated.

Figure 5 shows a point mutation in the cVO14 construct in a conserved region 5' to the OT gene. A conserved G residue is substituted with an A residue in the construct.

Figure 6 is an alignment of the sequences of 5'OT-EST from rat, human and mouse sources.

Figure 7 is a comparison of the body weights of transgenic and non-transgenic rats.

Figure 8 is a comparison of the body weights of transgenic and non-transgenic male and femnale rats.

Figure 9 is a comparison of the measurements (in mm) of the pelvis (b) and of the body length (a) of 20 and 52 week male transgenic and non-transgenic rats (mean +/- sem, ***=$p \leq 0.001$, n=6-7 per group).

Figure 10 illustrates the increased body weight/body length ratio of transgenic rats compared with non-transgenic rats.

Figure 11 shows the weights of the peri-renal and testicular fat pads in JP17 male transgenic and non-transgenic animals at different ages. (*=$p<0.05$; **=$p<0.01$, n=6-7 per group).

Figure 12 shows the levels of plasma insulin, glucose, cholesterol, triglycerides, leptin and corticosterone in terminal blood samples from transgenic and non-transgenic rats. Values shown are mean of each group +/- SEM (n=6 for transgenic groups; n=4 for non-transgenic groups) (* Significantly different ($p<0.05$) from sex matched non transgenic group).

Figure 13 shows the changes in body weight, leptin levels and food intake associated with young (100 day old) rats fed on normal fat (4%) or high fat (30%) diets. Results are shown for SLOB, non-transgenic and dwarf rats, fed either 4% fat diet (clear bars) or a 30% fat diet (stippled bars) over a 27-day period (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, high vs. low fat diet: ## = $p<0.01$; ### = $p<0.001$, SLOB vs. non-trnasgenic rats).

Figure 14 shows the changes in body weight associated with ovariectomy in transgenic rats and non-transgenic littermates. ■ - ovariectomised SLOB rat; □ - ovariectomised wild-type rat; • - sham ovariectomised SLOB rat; o - sham ovariectomised wild-type rat.

Detailed Description of the Invention

Definitions

As referred to herein, "5'OT-EST" is the polypeptide represented in SEQ. ID. Nos. 2, 4 or 6 (rat, human and mouse respectively). Preferably, it is the human sequence. However, the term also covers alternative peptides homologous to 5'OT-EST, such as polypeptides derived from other species, including other mammalian species.

"Mutants" of 5'OT-EST include polypeptides which differ only in minor, insignificant ways from wild-type 5'OT-EST, for example polypeptides having conservative amino acid replacements or additions or deletions. Preferred, however, are mutants which are able to confer, on animals expressing them, an obese phenotype as defined herein. An example of such a mutant is the 5'OT-EST - xdel polypeptide set forth in SEQ. ID. No. 8. Further mutants may be obtained as described herein, and defined according to their functional effects in transgenic animals or host cells.

"Substantially homologous", whether applied to polypeptide or nucleotide sequences, is as defined herein with reference to homology screening. It may be interpreted as referring either to sequence alignment and direct comparison, or to homology as defined by BLAST homology searching as defined herein.

A "transgenic animal" is an animal whose genome has been functionally altered by genetic manipulation. In the context of the present invention, this includes animals bearing and expressing a *5'OT-EST* or mutant *5'OT-EST* transgene, animals from which *5'OT-EST* sequences have been deleted or in which they have been modified, and animals which are transiently transformed to express a (mutant) *5'OT-EST* transgene such as by transformation with viral sequences.

"Transformation" refers to the functional insertion of a gene by nucleic acid transfer, or the functional deletion of a gene, in a cell or organism. The term thus includes transfection, transduction and any other techniques useful for transferring nucleic acids

into cells or organisms. Cells transformed according to the invention express a novel genotype as a result of the transformation.

For the avoidance of doubt, unless otherwise required by the specific context, reference herein to an entity in the singular includes the plural thereof. Thus, the expressions "a gene" and "one or more genes" are equivalent.

Moreover, unless otherwise required by context, references to 5'OT-EST (*5'OT-EST*) preferably include mutants of 5'OT-EST (*5'OT-EST*).

A "cosmid" is a bacteriophage-based vector as commonly known in the art.

References herein to "obesity" and obese animals are preferably references to the SLOB phenotype observed in SLOB rats according to the invention, characterised in being *inter alia* male-specific, late onset, with fat deposition concentrated in the abdominal area and associated with sterility.

Description of Preferred Embodiments

A cosmid (cVO14) of rat DNA containing the rat vasopressin (AVP) and rat oxytocin (OT) genes (Ivell & Richter, 1984) was constructed, and DNA reporter sequences inserted therein using standard methods (Sambrook *et al.*, 1989) as outlined in Examples 1 & 2 below. Microinjection of the cVO14 DNA insert into fertilised rat eggs and their transfer into pseudopregnant recipients resulted in production of viable offspring. Unexpectedly, the male founder rat with 4-5 copies of cVO14 (JP17) showed a dominant phenotype of severe late-onset visceral obesity. This form of obesity shows (i) a very late onset, (ii) a highly selective visceral distribution of fat developing on a normal rodent diet, without hyperphagia, (iii) an effect greatly preponderant in males, (iv) a predisposition to excessive dietary-fat induced obesity at an early age, before the phenotype becomes apparent on a normal diet, and (v) a dominant pattern of inheritance. Moreover, male transgenics show severe infertility in males, whilst females are fertile. Rats bearing this transgene have been termed **SLOB** rats (for **S**evere **L**ate-onset **OB**esity). The symptoms

of obesity observed in SLOB rats all occur in several forms of human obesity, including that associated with human syndrome-X (Reaven *et al.*, 1988) for which a late-onset increase in abdominally distributed fat, affecting males much more severely than females (Gray *et al.* 1997) may be mimicked in the SLOB rat. Obvious causes, such as leptin deficiency or insulin resistance or overt Type 1 or 2 diabetes may be excluded.

Although the SLOB phenotype is preponderant in males, it may be markedly exacerbated in females by ovariectomy.

Mapping and analysis of cosmid DNA used to generate cVO14, revealed a putative gene, 5' of the OT locus. A fragment of this DNA was subcloned and sequenced. Analysis of this region of rat DNA enabled us to determine the location, orientation, partial exon structure and predicted protein product of a novel gene lying 5' of the OT gene in rat DNA. Further sequencing and analysis elucidated the structure of this gene, and provided additional sequence information for the cosmid DNA surrounding the known sequence of the OT and AVP genes. The novel rat gene is termed herein *5'OT-EST*, which encodes the 5'OT-EST polypeptide. The genomic sequence of *5'OT-EST* is given in SEQ. ID. No. 16.

A search of DNA and protein databases revealed no significant match to any known gene, but recognised partial matches to DNA sequences homologous to *5'OT-EST* in expressed sequence tag (EST) databases from rat, mouse and human DNA sources. These represent partial products of the rat gene, and of genes homologous to this novel rat gene, in mouse and human DNA. The predicted structures of four exons, termed w, x, y, z, and predicted protein sequences are highly conserved between these species. A partial match was noted to a human genomic DNA sequence alluded to, but not disclosed in White *et al.* PNAS 95:305-309 (1998), but deposited by them in Genbank (Accession no:AF036329) as a putative genomic fragment containing the human GnRH-II gene. The relationship between human *5'OT-EST* and human *GnRH-II* as described by White *et al.* is confirmed by the present work; however, there does not appear to be any such relationship in rats or mice. Homologous rat *GnRH-II* sequences cannot be recognised by sequence analysis in cVO14, which contains more than 10kb of rat DNA flanking *5'OT-EST*. Neither can any

homologous mouse *GnRH-II* sequence be identified by hybridisation or PCR studies in multiple mouse genomic clones which contain *5'OT-EST* and at least 50kb of flanking DNA. Thus, it appears highly unlikely that the *GnRH-II* sequence corresponds functionally with *5'OT-EST*.

In rats, *5'OT-EST* lies about 10kb downstream of the 3' exon of the protein tyrosine phosphatase receptor alpha (*Ptpra*) gene, and the intervening 10kb show no homology with *GnRH-II*. Additionally, mouse BAC clones containing *5'OT-EST* show no homology to *GnRH-II*. Thus, *GnRH-II* sequences are not adjacent to *5'OT-EST* in rats or mice, and neither *GnRH-II* nor *Ptpra* is present in the cosmid used to generate SLOB rats. Complete sequencing of the cosmid reveals no other novel genes.

Based on physical linkage to *Ptpra*, *Avp* and *Oxt*, *5'OT-EST* maps to the distal region of mouse chromosome 2, 7.32 cM from the centromere. *Ptpra* has itself been implicated in the control of insulin sensitivity, and both *5'OT-EST* and *Ptpra* lie within 0.21 cM of *mg*, another gene implicated in the suppression of obesity. In mouse, all three genes map to the same region as the mouse obesity locus *Mob5* (Encyclopaedia of the Mouse genome VII: Mouse Chromosome 2; (1998) Peters *et al.*, Mamm. genome 8 Spec No:S27-49). It is likely therefore that *5'OT-EST* contributes to the trait observed at this locus in mice.

Accordingly, the present invention provides 5'OT-EST polypeptide. 5'OT-EST according to the present invention may be mouse, rat or human 5'OT-EST, as well as variants of 5'OT-EST derivable from other species or by natural or artificial mutation of a *5'OT-EST* gene.

The variant provided by the present invention includes splice variants encoded by mRNA generated by alternative splicing of a primary transcript, amino acid mutants, glycosylation variants and other covalent derivatives of 5'OT-EST which retain the physiological and/or physical properties thereof. Exemplary derivatives include molecules wherein 5'OT-EST is covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Such a moiety may be a detectable moiety such as an enzyme or a

radioisotope. Further included are naturally occurring variants of 5'OT-EST found within a particular species, preferably a mammal. Such a variant may be encoded by a related gene of the same gene family, by an allelic variant of a particular gene, or represent an alternative splicing variant of 5'OT-EST.

Variants which retain common structural features can be fragments of 5'OT-EST. Fragments of 5'OT-EST comprise smaller polypeptides derived from therefrom. Preferably, smaller polypeptides derived from 5'OT-EST according to the invention define a single feature which is characteristic of 5'OT-EST as described in the present application.

Derivatives of 5'OT-EST also comprise mutants thereof, which may contain amino acid deletions, additions or substitutions. Thus, conservative amino acid substitutions may be made substantially without altering the nature of 5'OT-EST. Deletions and substitutions may moreover be made to the fragments of 5'OT-EST comprised by the invention.

Mutants of 5'OT-EST according to the present invention may possess properties different from those of naturally occurring 5'OT-EST. In particular, 5'OT-EST mutants may modulate the expression of native 5'OT-EST.

5'OT-EST mutants may be produced from a nucleic acid encoding 5'OT-EST which has been subjected to in vitro mutagenesis resulting e.g. in an addition, exchange and/or deletion of one or more amino acids. For example, substitutional, deletional or insertional variants of 5'OT-EST can be prepared by recombinant methods and screened for immuno-crossreactivity with the native forms of 5'OT-EST.

Preferably, 5'OT-EST according to the present invention has the sequence of SEQ. ID. No. 2 (rat), SEQ. ID. No. 4 (human) or SEQ. ID. No. 6 (mouse). Mutants possessing desired properties may be generated from these sequences, or isolated from natural sources, by a variety of techniques which assess the biological function of the 5'OT-

EST mutant. For example, nucleic acids encoding 5'OT-EST mutants may be used to generate transgenic animals and these animals assessed for indications of an obesity phenotype.

For example, the effects of mutant transgenes may be assessed by carcass analysis, measurement of growth, body weight, body fat distribution, as well as other measures of analytes in body fluids or tissues relevant to obesity in transgenic animals (Mathe, 1995; Shillabeer, 1992). These include, but are not limited to, cholesterol, triglycerides, fatty acids, lipoproteins, and other dietary constituents or metabolites, as well as metabolic hormones, such as leptin, insulin, glucagon, catecholamines or glucocorticoids. Other relevant parameters include cardiovascular measures (Reaven, 1988, Gray & Yudkin, 1997). These may include measures of systolic or diastolic blood pressure, cardiac output, or vascular resistance, together with morphological changes to organ systems known to be affected by cardiovascular or obesity disorders, such as heart, major or minor blood vessels, their muscle or endothelial layers, and their elasticity or fragility. See for example McNamee *et al.* (1994).

Similarly, parameters related to the infertility phenotype that may be measured, include, but are not limited to, testicular weight, volume, development, spermatogenesis, sperm number, motility or ability to fertilise oocytes. They may also include measures of testicular fluid production and constituents, as well as products of other accessory organs including seminal vesicles or prostate, as well as hormones, receptors, and proteins important in male sexual function, such as testosterone, LH, FSH, inhibin or activin. Other responses that may be affected include energy expenditure, physical activity, ingestive behaviour, excretory behaviour, or reproductive behaviour, or the organs, hormones or receptors commonly recognised to be associated with these physiological systems, their metabolism or morphological structure.

5'OT-EST as disclosed herein is a polypeptide composed of four exons, termed w, x, y and z (see SEQ. ID. No. 16). Advantageously, mutants of 5'OT-EST are mutated in, or preferably lack all or part of, the sequences encoded by one or more exons of *5'-OT-EST*. Preferably, mutants of 5'OT-EST lack, or are mutated in, all or part of the sequences

encoded by exons x, y and z of *5'-OT-EST.*

Preferably, the sequences encoded by exons x, y and z are deleted and those encoded by exon w partially deleted. Most preferably, the mutant is 5'OT-EST - xdel as described herein, for example in SEQ. ID. No. 8.

The fragments, mutants and other derivatives of 5'OT-EST preferably retain substantial homology with 5'OT-EST. As used herein, "homology" means that the two entities share sufficient characteristics for the skilled person to determine that they are similar in origin and function. Preferably, homology is used to refer to sequence identity. Thus, the derivatives of 5'OT-EST preferably retain substantial sequence identity with 5'OT-EST.

"Substantial homology", where homology indicates sequence identity, means more than 40% sequence identity, preferably more than 45% sequence identity and most preferably a sequence identity of 50% or more, as judged by direct best-fit sequence alignment and comparison.

Sequence homology (or identity) may moreover be determined using any suitable homology algorithm, using for example default parameters. Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail at http://www.ncbi.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference. The search parameters are defined as follows, and are advantageously set to the defined default parameters.

Advantageously, "substantial homology" when assessed by BLAST equates to sequences which match with an EXPECT value of at least about 7, preferably at least about 9 and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs

ascribe significance to their findings using the statistical methods of Karlin and Altschul (see http://www.ncbi.nih.gov/BLAST/blast_help.html) with a few enhancements. The BLAST programs were tailored for sequence similarity searching, for example to identify homologues to a query sequence. The programs are not generally useful for motif-style searching. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (1994).

The five BLAST programs available at http://www.ncbi.nlm.nih.gov perform the following tasks:

blastp compares an amino acid query sequence against a protein sequence database;

blastn compares a nucleotide query sequence against a nucleotide sequence database;

blastx compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database;

tblastn compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

tblastx compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

HISTOGRAM Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

DESCRIPTIONS Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page). See also EXPECT and CUTOFF.

ALIGNMENTS Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

EXPECT The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E in the BLAST Manual).

CUTOFF Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

MATRIX Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992). The valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

STRAND Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see http://www.ncbi.nlm.nih.gov). Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence (e.g., "NNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.

It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating that the statistical significance of any matches reported against the unfiltered query sequence should be suspect.

NCBI-gi Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at http://www.ncbi.nlm.nih.gov/BLAST.

Conventional BLAST serches of the publically available databases do not reveal any homology of the predicted protein product of *5'OT-EST* to any known protein. However, application of a more sophisitcated search algorithm, as described in Taylor *et al.*, 1998, identifies structural similarities to apolipoprotein E (ApoE) in its alpha-helical domains, but without any apparent LDL-receptor domain. Since ApoE is centrally involved in lipid metabolism and transport, a role for 5'OT-EST in cellular lipid handling is suggested.

Accordingly, the invention provides a method for identifying a candidate compound capable of influencing lipid transport, comprising the steps of:

a) contacting 5'OT-EST polypeptide with a candidate compound or compounds and determining which candidate compound or compounds is capable of interacting with 5'OT-EST;

b) optionally, testing candidate compounds which interact with 5'OT-EST in a transgenic animal according to the invention.

According to a further aspect of the present invention, there is provided a nucleic acid encoding 5'OT-EST or a mutant thereof. In addition to being useful for the production of recombinant 5'OT-EST protein, these nucleic acids are also useful as probes, thus readily enabling those skilled in the art to identify and/or isolate nucleic acid encoding 5'OT-EST and/or mutant 5'OT-EST. The nucleic acid may be unlabelled or labelled with a detectable moiety. Furthermore, nucleic acid according to the invention is useful e.g. in a method determining the presence of 5'OT-EST-specific nucleic acid, said method comprising hybridising the DNA (or RNA) encoding 5'OT-EST (or its complement) to test sample nucleic acid and determining the presence of *5'OT-EST*. In another aspect, the invention provides a nucleic acid sequence that is complementary to, or hybridises under stringent conditions to, a nucleic acid sequence encoding 5'OT-EST.

The invention also provides a method for amplifying a nucleic acid test sample comprising priming a nucleic acid polymerase (chain) reaction with nucleic acid corresponding to *5'OT-EST*, including the untranslated regions (or its complement).

In still another aspect of the invention, the nucleic acid is DNA and further comprises a replicable vector comprising the nucleic acid encoding 5'OT-EST operably linked to control sequences recognised by a host transformed by the vector. Furthermore the invention provides host cells transformed with such a vector and a method of using a nucleic acid encoding 5'OT-EST to effect the production of 5'OT-EST, comprising expressing *5'OT-EST* nucleic acid in a culture of the transformed host cells and, if desired, recovering 5'OT-EST from the host cell culture.

Isolated *5'OT-EST* nucleic acid includes nucleic acid that is free from at least one contaminant nucleic acid with which it is ordinarily associated in the natural source of *5'OT-EST* nucleic acid or in crude nucleic acid preparations, such as DNA libraries and the like. Isolated nucleic acid thus is present in other than in the form or setting in which it is found in nature. However, isolated 5'OT-EST encoding nucleic acid includes *5'OT-EST* nucleic acid in ordinarily 5'OT-EST-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature.

In accordance with the present invention, there are provided isolated nucleic acids, e.g. DNAs or RNAs, encoding 5'OT-EST, particularly mammalian 5'OT-EST, e.g. human 5'OT-EST, or fragments thereof. In particular, the invention provides a DNA molecule encoding 5'OT-EST, or a fragment thereof. By definition, such a DNA comprises a coding single stranded DNA, a double stranded DNA of said coding DNA and complementary DNA thereto, or this complementary (single stranded) DNA itself. An exemplary nucleic acid encoding 5'OT-EST is represented in SEQ ID Nos. 1, 3 and/or 5.

The preferred sequence encoding 5'OT-EST is that having substantially the same nucleotide sequence as the coding sequences in SEQ ID Nos. 1, 3 and/or 5, with the nucleic acid having the same sequence as the coding sequence in SEQ ID Nos. 1, 3 and/or

5 being most preferred. As used herein, nucleotide sequences which are substantially the same share at least about 90% identity. However, in the case of splice variants having e.g. an additional exon sequence homology may be lower. Homology is determined as described above.

The invention moreover provides nucleic acids encoding 5'OT-EST, comprising the gene *5'OT-EST* or variants thereof as defined herein. The nucleic acids of the invention, whether used as probes or otherwise, are preferably substantially homologous to the sequence of *5'OT-EST* as shown in SEQ ID Nos. 1, 3 and/or 5. The terms "substantially" and "homologous" are used as hereinbefore defined with reference to the 5'OT-EST polypeptide.

Preferably, nucleic acids according to the invention are fragments of the *5'OT-EST*-sequence, or derivatives thereof as hereinbefore defined in relation to polypeptides. Fragments of the nucleic acid sequence of a few nucleotides in length, preferably 5 to 150 nucleotides in length, are especially useful as probes.

Exemplary nucleic acids can alternatively be characterised as those nucleotide sequences which encode a 5'OT-EST protein, or which correspond to untranslated regions of *5'OT-EST,* and hybridise to the DNA sequences set forth SEQ ID Nos. 2, 4 and/or 6, or a selected fragment of said DNA sequence. Preferred are such sequences encoding 5'OT-EST which hybridise under high-stringency conditions to the sequence of SEQ ID Nos. 1, 3 and/or 5.

Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (Tm) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M Na+ at 65-68 °C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 Na+ pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

Moderate stringency refers to conditions equivalent to hybridisation in the above described solution but at about 60-62 °C. In that case the final wash is performed at the hybridisation temperature in 1x SSC, 0.1 % SDS.

Low stringency refers to conditions equivalent to hybridisation in the above described solution at about 50-52 °C. In that case, the final wash is performed at the hybridisation temperature in 2x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, *et al.*, eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York or Ausubel, *et al.*, eds. (1990) Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the probe also play a role.

Advantageously, the invention moreover provides nucleic acid sequence which are capable of hybridising, under stringent conditions, to a fragment of SEQ. ID. Nos. 1, 3, 5 or 7. Preferably, the fragment is between 15 and 50 bases in length. Advantageously, it is about 25 bases in length, preferably about 20 bases in length. For differentiating between mutant and wild type *5'OT-EST* by PCR reactions, 20mers are the preferred size, whilst for use as probes in, for example, Southern hybridisation, the use of 40mers is preferred. Riboprobes may be designed to be substantially any length, up to and including the entire length of the largest specific cDNA sequence.

Specifically included, moreover, are sequences complementary to the foregoing sequences.

Given the guidance provided herein, the nucleic acids of the invention are obtainable according to methods well known in the art. For example, a DNA of the invention is obtainable by chemical synthesis, using polymerase chain reaction (PCR) or by screening a genomic library or a suitable cDNA library prepared from a source believed to possess *5'OT-EST* and to express it at a detectable level.

Chemical methods for synthesis of a nucleic acid of interest are known in the art and include triester, phosphite, phosphoramidite and H-phosphonate methods, PCR and other autoprimer methods as well as oligonucleotide synthesis on solid supports. These methods may be used if the entire nucleic acid sequence of the nucleic acid is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

An alternative means to isolate the gene encoding 5'OT-EST is to use PCR technology as described e.g. in section 14 of Sambrook *et al.*, 1989. This method requires the use of oligonucleotide probes that will hybridise to *5'OT-EST* nucleic acid. Strategies for selection of oligonucleotides are described below.

Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries suitable means include monoclonal or polyclonal antibodies that recognise and specifically bind to 5'OT-EST; oligonucleotides of about 20 to 80 bases in length that encode known or suspected *5'OT-EST* cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a hybridising gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to oligonucleotides, cDNAs or fragments thereof that encode the same or hybridising DNA; and/or homologous genomic DNAs or fragments thereof.

A nucleic acid encoding 5'OT-EST may be isolated by screening suitable cDNA or genomic libraries under suitable hybridisation conditions with a probe, i.e. a nucleic acid disclosed herein including oligonucleotides derivable from the sequences set forth in SEQ ID Nos. 1, 3 and/or 5. Suitable libraries are commercially available or can be prepared e.g. from cell lines, tissue samples, and the like.

As used herein, a probe is e.g. a single-stranded DNA or RNA that has a sequence of nucleotides that includes between 10 and 50, preferably between 15 and 30 and most preferably at least about 20 contiguous bases that are the same as (or the complement of) an equivalent or greater number of contiguous bases set forth in SEQ ID Nos. 1, 3 and/or 5. The nucleic acid sequences selected as probes should be of sufficient length and sufficiently unambiguous so that false positive results are minimised. The nucleotide sequences are usually based on conserved or highly homologous nucleotide sequences or regions of *5'OT-EST*. The nucleic acids used as probes may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is not known.

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode ligand binding sites, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labelled with suitable label means for ready detection upon hybridisation. For example, a suitable label means is a radiolabel. The preferred method of labelling a DNA fragment is by incorporating $^{\alpha-32}$P dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labelled with $^{\gamma-32}$P-labelled ATP and polynucleotide kinase. However, other methods (e.g. non-radioactive) may also be used to label the fragment or oligonucleotide, including e.g. enzyme labelling, fluorescent labelling with suitable fluorophores and biotinylation.

Probes for cloning and amplifying *5'OT-EST*, especially human *5'OT-EST*, may be deduced from the sequence thereof provided herein. Preferred probes may be selected from the following:

1U	GGACAGCCCGAAGGACTACAGGT	SEQ. ID. No. 18
1L	CGAAGAACTCCGCAGGGTCC	SEQ. ID. No. 19
2U	AAGACCCGCCACGACCCG	SEQ. ID. No. 20
2L	GAATCAGCACCCTCTCCGCC	SEQ. ID. No. 21
3U	TGCGGAGTTCTTCGTGCTGATGGAG	SEQ. ID. No. 22
3L	GGTGCTCGGCGGCGTCCTTC	SEQ. ID. No. 23
4U	GAGTGGCGGAGAGGGTGCTGA	SEQ. ID. No. 24
4L	GGCCGAGGCTGAGCGGGG	SEQ. ID. No. 25
5U	CTGAAGGACGCCGCCGAGCA	SEQ. ID. No. 26
5L	CTCCAACGCCTGCCGCTGC	SEQ. ID. No. 27
6U	GCAGGAGGAGCGGGAGCAGGA	SEQ. ID. No. 28
6L	TCCAGTGCCCCGCAAGCCG	SEQ. ID. No. 29

Probes according to the invention are suitable for use as diagnostic reagents to amplify *5'OT-EST* and thereby enable the analysis of the nucleic acid for the presence of mutations, polymorphisms or other changes which could render an individual susceptible to obesity.

After screening the library, e.g. with a portion of DNA including substantially the entire 5'OT-EST-encoding sequence or a suitable oligonucleotide based on a portion of said DNA, positive clones are identified by detecting a hybridisation signal; the identified clones are characterised by restriction enzyme mapping and/or DNA sequence analysis,

and then examined, e.g. by comparison with the sequences set forth herein, to ascertain whether they include DNA encoding a complete 5'OT-EST (i.e., if they include translation initiation and termination codons). If the selected clones are incomplete, they may be used to rescreen the same or a different library to obtain overlapping clones. If the library is genomic, then the overlapping clones may include exons and introns. If the library is a cDNA library, then the overlapping clones will include an open reading frame. In both instances, complete clones may be identified by comparison with the DNAs and deduced amino acid sequences provided herein.

In order to detect any abnormality of endogenous *5'OT-EST*, genetic screening may be carried out using the nucleotide sequences of the invention as hybridisation probes or as PCR primers, using which genomic nucleic acid may be amplified, and subsequently sequenced. Also, based on the nucleic acid sequences provided herein antisense-type therapeutic agents may be designed.

It is envisaged that the nucleic acid of the invention can be readily modified by nucleotide substitution, nucleotide deletion, nucleotide insertion or inversion of a nucleotide stretch, and any combination thereof. Such mutants can be used e.g. to produce a 5'OT-EST mutant that has an amino acid sequence differing from the 5'OT-EST sequences as found in nature. Mutagenesis may be predetermined (site-specific) or random. A mutation which is not a silent mutation must not place sequences out of reading frames and preferably will not create complementary regions that could hybridise to produce secondary mRNA structure such as loops or hairpins.

The invention accordingly specifically includes nucleic acids encoding mutants of 5'OT-EST, as defined above. Such nucleic acids may be used for all the purposes identified above in relation to wild-type *5'OT-EST* nucleic acids. Particularly preferred are nucleic acids encoding *5'OT-EST - xdel*, which preferably have the sequence ATGTTGCGGGCTTTGAACCGCCTGGCCGCGCGGCCCGGGGGCCAGCCCCCAACCCTGCTC CTTCTGCCCGTGCGCGGCCCACGGCCCCGCTCATTCTCGGCTCCTTTTTCCTCGCAGGAT AGC (see SEQ. ID. No. 7), or an equivalent sequence which encodes the same polypeptide having regard to the degeneracy of the nucleic acid code, or a sequence

substantially homologous thereto or complementary thereto. In *5'-OT-EST - xdel* exon x is deleted, and exons y and z are out of frame and therefore not translated.

For hybridisation probes, it may be desirable to use nucleic acid analogues, in order to improve the stability and binding affinity. A number of modifications have been described that alter the chemistry of the phosphodiester backbone, sugars or heterocyclic bases.

Among useful changes in the backbone chemistry are phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulphur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH2-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids replace the entire phosphodiester backbone with a peptide linkage.

Sugar modifications are also used to enhance stability and affinity. The α-anomer of deoxyribose may be used, where the base is inverted with respect to the natural β-anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to degradation without comprising affinity.

Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and 5-propynyl-2'-deoxycytidine have been shown to increase affinity and biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

The DNA sequences, particularly nucleic acid analogues as described above, may be used as antisense sequences.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described nucleic acids. Such host cells such as prokaryote,

yeast and higher eukaryote cells may be used for replicating DNA and producing 5'OT-EST. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, such as E. coli, e.g. E. coli K-12 strains, DH5α and HB101, or Bacilli. Further hosts suitable for 5'OT-EST encoding vectors include eukaryotic microbes such as filamentous fungi or yeast, e.g. Saccharomyces cerevisiae. Higher eukaryotic cells include insect and vertebrate cells, particularly mammalian cells, including human cells, or nucleated cells from other multicellular organisms. The propagation of vertebrate cells in culture (tissue culture) is a routine procedure. Examples of useful mammalian host cell lines are epithelial or fibroblastic cell lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 cells, HeLa cells or 293T cells. The host cells referred to in this disclosure comprise cells in *in vitro* culture as well as cells that are within a host animal.

DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene to monitor transfection efficiency.

To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient amount of 5'OT-EST-encoding nucleic acid to form 5'OT-EST. The precise amounts of DNA encoding 5'OT-EST may be empirically determined and optimised for a particular cell and assay.

Host cells are transfected or, preferably, transformed with the above-captioned expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation

technique or by electroporation. Numerous methods of transfection are known to the skilled worker in the field. Successful transfection is generally recognised when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g. Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press).

Transfected or transformed cells are cultured using media and culturing methods known in the art, preferably under conditions, whereby 5'OT-EST encoded by the DNA is expressed. The composition of suitable media is known to those in the art, so that they can be readily prepared. Suitable culturing media are also commercially available.

The cDNA or genomic DNA encoding native or mutant 5'OT-EST can be incorporated into vectors for further manipulation. As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the artisan. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, i.e. whether it is to be used for DNA amplification or for DNA expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and optionally a signal sequence.

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (e.g. SV 40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another class of organisms for expression. For example, a vector is cloned in E. coli and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion into the host genome. However, the recovery of genomic DNA encoding 5'OT-EST is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise *5'OT-EST* DNA. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in E. coli, an E. coli genetic marker and an E. coli origin of replication are advantageously included. These can be obtained from E. coli plasmids, such as pBR322, Bluescript© vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both E. coli replication origin and E. coli genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up *5'OT-EST* nucleic acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes 5'OT-EST. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to *5'OT-EST* nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to DNA encoding 5'OT-EST by removing the promoter from the source DNA by restriction enzyme

digestion and inserting the isolated promoter sequence into the vector. Both the native *5'OT-EST* promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of *5'OT-EST* DNA. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Promoters suitable for use with prokaryotic hosts include, for example, the β-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them to DNA encoding 5'OT-EST, using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the DNA encoding 5'OT-EST.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phagex or T7 which is capable of functioning in the bacteria. In one of the most widely used expression systems, the nucleic acid encoding the fusion protein may be transcribed from the vector by T7 RNA polymerase (Studier *et al.*, Methods in Enzymol. 185; 60-89, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the λ-lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible lac UV5 promoter. This system has been employed successfully for over-production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int- phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) , or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (new England Biolabs, MA, USA).

Moreover, the *5'OT-EST* gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a soluble native peptide rather than in an inclusion body. The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a Saccharomyces cerevisiae gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the a- or α-factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, the *S. cerevisiae* GAL 4 gene, the *S. pombe* nmt 1 gene or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PHO5 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

5'OT-EST gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian promoters such

as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from the promoter normally associated with *5'OT-EST* sequence, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding 5'OT-EST by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to *5'OT-EST* DNA, but is preferably located at a site 5' from the promoter.

Advantageously, a eukaryotic expression vector encoding 5'OT-EST may comprise a locus control region (LCR). LCRs are capable of directing high-level integration site independent expression of transgenes integrated into host cell chromatin, which is of importance especially where the *5'OT-EST* gene is to be expressed in the context of a permanently-transfected eukaryotic cell line in which chromosomal integration of the vector has occurred, in vectors designed for gene therapy applications or in transgenic animals.

Eukaryotic expression vectors will also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding 5'OT-EST.

An expression vector includes any vector capable of expressing *5'OT-EST* nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or

other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. For example, DNAs encoding 5'OT-EST may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, *et al.*, (1989) NAR 17, 6418).

Particularly useful for practising the present invention are expression vectors that provide for the transient expression of DNA encoding 5'OT-EST in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high levels of 5'OT-EST. For the purposes of the present invention, transient expression systems are useful e.g. for identifying 5'OT-EST mutants, to identify potential phosphorylation sites, or to characterise functional domains of the protein.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing 5'OT-EST expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe which may be based on a sequence provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

In a further aspect, the present invention provides a transgenic non-human animal which

expresses, as a result of transformation with a transgene, 5'OT-EST or a mutant thereof as defined herein. Preferred animals include mammals, especially rats.

Preferably, the non-human animal is a mammal suitable for use as a test system for therapies and treatments relating to obesity, including human obesity and animal obesity, which is of concern in household animals such as cats and dogs. Thus, the mammal may be a cat or a dog, or other household pet; it is preferably a rodent, such as a mouse or a rat, particularly a rodent adapted for laboratory testing whose genotype and general characteristics are well known.

Any technique may be used to generate transgenic animals according to the invention. Preferably, the technique involves transfer of a transgene comprising *5'OT-EST* to the pronucleus of a single-cell embryo, prior to implantation of the embryo into a pseudopregnant foster mother. Such techniques have the advantage that germ-line transgenic animals are readily produced.

Alternatively, transgenic animals may be created by ES cell transfer techniques. In such techniques, ES cells are transformed with the desired transgene and then used to reconstitute an embryo. Animals created by such techniques are normally chimeric for the transgene. However, more accurate positional insertion of the transgene is possible, and selective deletion of endogenous genes by homologous recombination is facilitated (Mansour *et al.*, 1989).

Further techniques include targeted or non-targeted delivery of genes to whole animals, using viral or non-viral vectors. For example, genes may be delivered by recombinant retroviruses or adenovius vectors, including adeno-assisted virus vectors, which are capable of integrating into the genome of the animal and expressing the delivered gene. Non-viral vectors include liposomal vectors, antibody-targeted DNA-protein complexes and the like.

As used herein, "transgenic" animals include animals from which *5'OT-EST* has been deleted, as well as animals to which a *5'OT-EST* transgene has been added. Optionally,

the endogenous *5'OT-EST* may be deleted, and a transgene bearing a heterologous or homologous *5'OT-EST* gene, which may be wild-type or mutated, inserted into the animal.

Preferred vectors for creating transgenic animals include linearised naked DNA from a variety of sources. In a preferred embodiment, transgenes may be derived from linearised cosmid sequences, from which the phage-related sequences have optionally been removed.

The *5'OT-EST* sequences used in a transgene according to the present invention may be inserted separately, or together with further sequences, including reporter genes, further effector genes and the like. Preferably, *5'OT-EST* is comprised in a nucleic acid fragment which comprises the natural wild-type environment of *5'OT-EST*, including flanking sequences.

5'OT-EST is located proximal to the vasopressin (AVP) and oxytocin (OT) genes in the genome, being transcribed in opposite directions from positions closely linked in a single locus. *5'OT-EST* lies 5' of the OT gene in at the OT/AVP locus. Accordingly, the transgene preferably consists of the OT/AVP locus, including *5'OT-EST*. Advantageously, one or more of the OT, AVP and *5'OT-EST* genes may be mutated, for example by insertion of reporter genes, such as the hGH gene.

In a highly preferred aspect, the transgene is cosmid cVO14 as described in Figure 4 herein. The complete sequence of cVO14 is set forth in SEQ. ID. No. 17.

Transgenic animals according to the invention may comprise single copies of the transgene, or may comprise multiple integrated copies, which may be present as concatamers. Preferably, transgenic animals according to the invention comprise four or more copies of the transgene.

Transgenic animals according to the invention may be employed for a variety of purposes. The characteristics of male specificity, central distribution of adiposity, late onset and

severity, and associated morbidity have parallels in the description of several human forms of central obesity. These include, but are not limited to, the condition known as metabolic syndrome, or Syndrome X, as well as other forms of central obesity which may be most severely expressed in human males, with or without reduced fertility and which are associated with increased morbidity. (For recent reviews of the importance of clinical and health care issues in obesity see Science 1998, vol. 280 pp. 1364-1390). Transgenic animals expressing *5'OT-EST* or mutants thereof thus have particular beneficial utility as a novel animal model of late-onset human visceral obesity, preponderant in males.

Moreover, the induction of the SLOB phenotype in juvenile rats as a result of dietary fat increases suggests that transgenic animals expressing *5'OT-EST* are a model for juvenile obesity in mammals, predominantly male mammals, which is induced by the consumption of a high-fat diet.

Furthermore, the onset of obesity in ovariectomised SLOB female rats suggests the model may be suitable to investigate post-menopausal obesity in female mammals.

For instance, one recognised value of animals bearing *5'OT-EST* or mutant constructs is to use such animals and their nontransgenic littermates as animal experimental models for studying obesity or male infertility and their related conditions. Using the information disclosed herein, it is possible to identify transgenic animals before they become obese or sexually mature, and to use them as a model for studying the factors that affect the development of obesity or male infertility in any animal classified as a mammal, including humans, domestic, and farm animals, and zoo, sports, or pet animals, such as but not limited to sheep pigs, cows , horses, dogs, cats, *etc*.

In particular, rodent models of obesity or infertility are of value in testing the ability of pharmaceutical preparations of novel agents, to be beneficial in delaying or preventing the occurrence, development, course, severity, progression, or exacerbation of obesity or infertility (Mathe, 1995; Fan *et al.*, 1997). Animals bearing *5'OT-EST* or mutant constructs are particularly useful in testing agents in this regard, since the phenotype is predictable and non-transgenic littermates are ideal controls.

In addition to screening for unknown compounds, animals bearing *5'OT-EST* or mutants thereof may be particularly useful in studies employing administration of natural or recombinant proteins, peptides or other agents or their derivatives already known or suspected to be involved in some forms of obesity or male infertility (e.g. growth hormones, or reproductive hormones, their homologues, analogues, antagonists, inhibitors or secretagogues, or leptin, its homologues, analogues and antagonists) or other natural or pharmacological agents already known to be active and/or of therapeutic value in these conditions (e.g. insulin, thiazolidinones, catecholamines, gonadal steroids) or agents already known to affect their actions, distribution, catabolism or elimination).

Typically in such studies, compounds may be administered to animals bearing *5'OT-EST* or mutants thereof and their non-transgenic littermates by oral, parenteral (e.g., intramuscular, intraperitoneal, intravenous, or subcutaneous injection or infusion, or implant), nasal, pulmonary, rectal, sublingual, or topical routes of administration, and can be formulated in dosage forms appropriate for each route of administration, e.g. in soluble form, suspension, or other suitable pharmaceutical formulations.

For example, the effects of such compounds on the obese phenotype may be assessed by carcass analysis, measurement of growth, body weight, body fat distribution, as well as other measures of analytes in body fluids or tissues relevant to obesity (Mathe, 1995; Shillabeer, 1992). These include, but are not limited to, cholesterol, triglycerides, fatty acids, lipoproteins, and other dietary constituents or metabolites, as well as metabolic hormones, such as leptin, insulin, glucagon, catecholamines or glucocorticoids. Other relevant parameters include cardiovascular measures (Reaven, 1988, Gray & Yudkin, 1997). These may include measures of systolic or diastolic blood pressure, cardiac output, or vascular resistance, together with morphological changes to organ systems known to be affected by cardiovascular or obesity disorders, such as heart, major or minor blood vessels, their muscle or endothelial layers, and their elasticity or fragility. See for example McNamee *et al.* (1994).

Similarly, parameters related to the infertility phenotype that may be measured, include,

but are not limited to, testicular weight, volume, development, spermatogenesis, sperm number, motility or ability to fertilise oocytes. They may also include measures of testicular fluid production and constituents, as well as products of other accessory organs including seminal vesicles or prostate, as well as hormones, receptors, and proteins important in male sexual function, such as testosterone, LH, FSH, inhibin or activin. Other responses that may be affected include energy expenditure, physical activity, ingestive behaviour, excretory behaviour, or reproductive behaviour, or the organs, hormones or receptors commonly recognised to be associated with these physiological systems, their metabolism or morphological structure.

Compounds identified as effective in such screening or analysis based on the use of animals bearing *5'OT-EST* or mutants thereof are particularly useful in treatment of late-onset visceral obesity, or male infertility, in particular where they occur in combination, and disorders related to these conditions with a view to delaying or preventing the occurrence, development, course, severity or progression of the phenotype, avoiding its exacerbation, and preferably promoting its amelioration or cure in animals of commercial importance, or more preferably in humans.

In another embodiment, converse but also therapeutically valuable compounds may be developed based on screening or analysis as above in animals bearing *5'OT-EST* or mutants thereof but which are intended to *promote* the occurrence, development, or progression of *increased* fat deposition or increased calorie intake or decreased energy consumption, Such disorders in humans include, but are not limited to, wasting, or anorexia, or cachexia, associated with prolonged illness, or malabsorptive states or catabolic states associated with other diseases, such as, but not limited to, inflammatory conditions, Crohns disease, or AIDS wasting, or burns, or cancer, or bone disease.

Similarly, therapeutically valuable approaches may be developed based on screening or analysis as above in animals bearing *5'OT-EST* or mutants thereof but which *reduce* the degree of male fertility in those conditions in which it might be beneficial. For example, this may be beneficial in the control of populations in animals of commercial or environmental importance, or to develop novel forms of contraception which may be

effective in human males. Such approaches specifically include, but are not limited to, the possibility of blocking *5'OT-EST* or mutants thereof function by administering *5'OT-EST* mutant products or by immunisation against *5'OT-EST* to generate neutralising antibodies that interfere with the normal functioning of this gene product in the testis, or hypothalamus or adrenal gland or gastrointestinal tract, or other organ system in which *5'OT-EST* is expressed or upon which its products act.

The development and late-onset of obesity in transgenic animals may be particularly useful in studying the chronic effects of novel food additives or formulations designed to prevent or exacerbate the deposition of fat in animals of commercial importance, of destined for use in human food products or dietary aids. Such compounds may be administered as above and their effects on the development, course, severity, progression, exacerbation, amelioration or cure of the obesity phenotype assessed as described above. Additives or formulations shown to reduce the development of visceral obesity in this model may have utility in human food products or dietary aids or find beneficial medicinal use in reducing fat accretion or retention.

In another embodiment, transgenic animals, such as mice bearing *5'OT-EST* or mutants thereof or in which *5'OT-EST* has been disrupted, may be usefully intercrossed with other animal strains with defined mutations, or with undefined genetic backgrounds associated with propensity for the development of obesity or infertility. Comparison of the resulting progeny with or without the *5'OT-EST* transgene will provide additional information on the alterations in occurrence, development, course, severity, progression, exacerbation, amelioration or cure of the obesity phenotype when expressed in these other genetic backgrounds, and analysed as described above. Such intercrossing may then be envisaged to enhance the utility of the resulting progeny exhibiting the obesity phenotype.

Examples of this use include (without being limited to) interbreeding with Zucker fa/fa rats (Iida *et al.*, 1996), corpulent (cp) rats (Kahle *et al.*, 1997), OLETF rats (Takiguchi *et al.*, 1998), ZDF rats, tfm rats, spontaneously hypertensive or salt-sensitive rats (Michaelis *et al.*, 1995) or other dwarf rats such as dw/dw (Charlton *et al.*, 1988) or dr/dr rats (Takeuchi *et al.*, 1991). An example of the utility of this approach is given by

(Michaelis *et al.*, 1995). Examples of mouse lines that may be usefully interbred with mice carrying transgenes or deletions affecting *5'OT-EST* include, but are not limited to, ob/ob. db/db, tfm/tfm or hpg/hpg mice. A related example includes intercrossing mice carrying transgenes or deletions affecting *5'OT-EST* with other strains of mice in which genes already known to be involved in obesity or male fertility have been deleted by homologous recombination or introduced by transgenesis (singly or in combination). Examples of these are already known to include (but are not limited to) leptin, tubby and related genes, NPY, insulin, GLP-1, IGF-1, IGF-II, MCH, CRH, POMC, CCK, orexins or hypocretins, CART peptides, agouti protein, as well as the genes or alternate products structurally related to or homologous with, the above peptides. This example is also intended to include mice with disruptions in or extra copies of normal or mutated forms of, genes for the specific receptors of the peptides listed above (for example NPY receptors, such as subtype 5), or bombesin-receptor 3, IRS-1 or 2, uncoupling proteins such as UCP1-3, carboxypeptidase E, or PPARs or adrenergic receptor subtype 3 or TNF alpha or, all of which have been implicated in obesity.

Similarly, the fertility disruption in transgenic animals according to the invention may also be studied to advantage by crossing these animals or other animals in which *5'OT-EST* has been disrupted onto genetic backgrounds in which genes for gonadal or adrenal steroid biosynthesis or metabolism or gonadal steroid receptors and other reproductive hormones or their receptors or hypothalamic or pituitary hormones thought to affect male fertility (such as gonadotrophins, activins, inhibins, PRL, GnRH or transcription factors such as DAX1 or SF1, or other known gene products affecting male gonadal development, such as MIS, AMH, SCF) have been disrupted. Comparison of the resulting progeny with or without the *5'OT-EST* or mutant transgene may shed light on the alterations in occurrence, development, course, severity, progression, exacerbation, amelioration or cure of the infertility phenotype when expressed in these other genetic backgrounds. Such intercrossed lines, for example with those genetic strains as outlined above, and in which the obesity phenotype is present in full or in a modified form, may also be additionally useful for screening applications.

Conversely, the transfer of the obese phenotype onto these genetic backgrounds may also

alter the occurrence, development, course, severity, progression, exacerbation, amelioration or cure of the specific phenotypes expressed in the strain with which animals bearing *5'OT-EST* or mutants thereof are bred. Such intercrossed lines, for example with those genetic strains as outlined above, and in which the obesity phenotype is present in full or in a modified form, may also be useful for screening applications.

Animals bearing *5'OT-EST* or mutants thereof may be used to study the transfer of other gene products other than by breeding, e.g. by administration of suitable vectors containing constructs expressing proteins of interest, or by transgenesis. Such examples include, but are not limited to, constructs containing the gene products or analogues of other genes already thought to be active in obesity or male infertility, whose effects may be advantageously studied in transgenic animals according to the invention due to the predictable development of their phenotype. Such genes and their products include those mentioned in above in relation to alternative obese strains. Such derived animals in which the obesity phenotype is present in full or in a modified form, may also be useful for the various applications outlined above.

Animals bearing *5'OT-EST* or mutants thereof and exhibiting a specific late-onset visceral obesity may prove of particular value when used in a similar way to screen for the beneficial effects of reducing or eliminating other gene products by their silencing or elimination as described above using transgenesis, or homologous recombination, or by adenoviral delivery of antisense nucleotides. Examination of any alterations in the occurrence, development, course, severity, or progression of the obesity phenotype in these genetic backgrounds would be of utility in identifying the role, if any, of such disrupted genes in the expression of the obesity phenotype. Such animals in which the obesity phenotype is present in full or in a modified form, may also be useful for the applications outlined above.

In another embodiment, animals bearing *5'OT-EST* or mutants thereof or material derived from them and/or their nontransgenic littermates may prove useful in experiments designed to identify obesity -related or male-fertility-related differences in gene expression, RNA transcripts, proteins, or other biochemical measures, such as, but not

limited to lipids, peptides, carbohydrates, amino acids or compounds or precursors or metabolites thereof, or their distribution, in whole animals, or in samples of biological fluids taken from animals bearing *5'OT-EST* or mutants thereof. These may include, but are not limited to: serum, plasma, lymph fluid, synovial fluid, follicular fluid, seminal fluid, amniotic fluid, milk, whole blood, urine, cerebrospinal fluid, saliva, sputum, tears, perspiration, mucus, tissue culture medium, tissue extracts, and cellular extracts.

Similar analyses may be advantageously performed in samples of any tissue from animals bearing *5'OT-EST* or mutants thereof or their non-transgenic littermates, or tissue derived from animals interbred with SLOB rats or other animals bearing *5'OT-EST* or mutants thereof. Such tissues are preferably (but not limited to) endocrine tissues, such as pancreas, adrenals, or pituitary gland , adipose tissues from different locations, preferably but not limited to, inguinal, omental, perirenal, subcutaneous, mammary, periorbital or other regions, thermogenic fat, brown or white adipose tissue in other locations, areas of the CNS though to be involved in obesity, preferably but not limited to the hypothalamus, and other tissues, preferably, but not restricted to liver, gastrointestinal tract, gonads, heart, musculoskeletal system, immune system, kidney, connective tissue including skin, epithelial or endothelial tissues.

Specifically included are cells or tissues removed from animals bearing *5'OT-EST* or mutants thereof, or animals interbred with them, and maintained thereafter ex vivo, e.g. in tissue culture, or by transplantation in animals bearing *5'OT-EST* or mutants thereof or other hosts, with or without immune suppression, provided the particular utility is enhanced by the presence of the obesity gene or phenotype.

The invention thus provides the use of a tissue derived from a transgenic animal according to any one of claims 17 to 24 in a screen to identify a genetic cause of obesity, comprising the steps of:

a) isolating one or more gene products from tissue derived from a transgenic animal according to any one of claims 17 to 24; and

b) determining whether the expression of a gene product is correlated with obesity.

Tissues derived from SLOB rats, including in particular fat pad tissue, may for example be used for differential screening in order to determine differences in gene expression between obese and non-obese animals. The gene products analysed may be nucleic acid or protein gene products. For example, mRNA may be isolated from the tissue and screened to identify differentially expressed transcripts. In particular, gene products which may be involved in cellular lipid transport may be identified by such means.

The development of obesity or male infertility itself in animals bearing *5'OT-EST* or mutants thereof is predicted to induce secondary changes in other obesity or fertility - related parameters and regulators. These include, but are not limited to, blood pressure, pituitary hormones, sperm development, maturation, and/or motility, lipid mobilising enzymes or receptors, or agents controlling these. The latter include, but are not limited to leptin and its receptors, melanocortin, NPY, catecholamines, adrenal or gonadal or pituitary hormones, gut hormones such as insulin and glucagon, growth hormone and other growth factors such as members of the GH and IGF-1 families, their binding proteins and receptors. They may also include drugs of several classes that have be thought useful in obesity. Examples of such classes include agents affecting the serotonin system or the fat cell free fatty acid uptake or release or metabolism or lipase activity or hepatic lipid uptake, or insulin sensitisers. This example may also include morphological alterations in any tissue or cells of the cardiovascular system, including but not limited to, the heart and major blood vessels, other blood vessels carrying either arterial or venous blood, and any or all cells comprising these tissues.

Transgenic animals according to the invention appear to present with obesity without obvious diabetes or hypercortisolism. They may thus prove particularly beneficial in studying the developmental changes in these secondary parameters induce by other means, in the development of diabetes, or hypertension or cardiovascular disease or hypercortisolism (Russell *et al.*, 1993), all which are known to be associated with obesity in humans (Reaven, 1988). Examples of such means includes (but is not limited to) variation in dietary components or quantity, or treatment with diabetogenic agents, such as GH or cortisol. Examples of such agents affecting cardiac output or peripheral resistance or blood pressure include angiotensin-converting enzyme inhibitors or cardiac

glycosides, or beta adrenergic receptor 3 agonists or antagonists.

Morphological changes may also be seen in adipose tissues or cells, or the other tissues in the body containing fat, such as the liver and related cells, or the skeleton, or in other organs or tissues in the gonadal system, relating to the effects on male fertility. Differences in these measures detected specifically in animals bearing *5'OT-EST* or mutants thereof and their alteration by elimination, blockade, endogenous stimulation, or exogenous administration of anti-obesity or other agents affective in obesity or male infertility or related disorders would provide novel approaches to evaluate improve and perfect existing or novel therapeutic approaches to obesity or male infertility in other animals of commercial importance, and more preferably, in humans. An obvious example is the ready source of adipocyte cells and products from specific fat depots that are differentially increased in transgenic animals according to the invention. Responses to agents affecting fat cell metabolism or fat storage or lipogenesis or lipolysis or lipid-lowering agents, may be studied with particular advantage to discern effects on visceral or peripheral fat tissues. and to seek differential effects on fat from different depots in the animals.

The information disclosed herein will enable those skilled in the art to produce protein or peptide fragments corresponding in sequence to 5'OT-EST or mutants thereof, as described above. Such proteins or peptides (or simple analogues thereof), when administered to rats or other animals, or more preferably humans, would be expected to affect the development of obesity and fertility in males, and serve as the basis for the development of similar compounds, based on the homologous human sequences, useful in the treatment of these conditions in humans.

This also includes simple analogues that incorporate alterations known to improve the in vivo stability or delay the clearance, elimination or metabolism of proteins or peptides such as those derived from *5'OT-EST* or mutants thereof. Such alterations are obvious to those skilled in the art, and examples include amidation or acetylation of C or N-termini of peptides, or replacement of methionine residues with norleucine residues to avoid oxidation. This example also includes formulations or modifications of proteins known

to be effect for the same purposes, e.g. by PEGylation to prolong the half-life of peptides or proteins, or formulations of proteins with inert carriers (such as mannitol or lactose) or buffers or salts, that provide stable solutions suitable for in vivo administration of the active agents to animals or to humans.

In another embodiment, the information disclosed herein will enable those skilled in the art to design nucleotide probes for, or develop polyclonal or monoclonal antibodies against, the DNA, RNA or protein sequences corresponding to the whole or parts of the *5'OT-EST* gene in other animals of commercial importance, or more preferably, humans. These are of value in diagnostic tests to screen for mutations in this gene in animal or human populations subject to variations in obesity or fertility. They may also be used to monitor the development, progression, amelioration or cure of obesity or infertility as may be reflected in changes in the activity of this gene or its products. Such predictive tests are recognised to have beneficial value when applied to the human population (Whitaker *et al.*, 1997)

Examples of such probes or peptides or proteins used to develop antibodies include those predicted from the wild-type and mutated sequences in the rat *5'OT-EST* gene and mutants thereof, as well as their derivatives as described above, as well as those that may be inferred from homologous genes in human and mouse, either as intact sequences or formed in whole or in part as fusion sequences with other proteins to facilitate production or purification by standard methods known to those skilled in the art. For this purpose, products of the *5'OT-EST* gene may also be reacted with, produced as fusion products with, or mixed with, other proteins or adjuvants known to enhance the immune response. Also included are modifications to the nucleotide sequences, already known to those skilled in the arts to confer useful chemical properties on the products, for example by incorporating modified nucleotides to render them more stable, or to incorporate nucleotides tagged with functional groups such as biotin or digoxigenin, or incorporation of fluorescent or radioisotopically labelled derivatives, in order to render the products themselves more readily detectable.

In a further embodiment, the information disclosed herein will enable those skilled in the

art to isolate factors which interact directly with 5'OT EST or mutants thereof. For example, two-hybrid screens provide a means for isolating genes for proteins which interact with 5'OT EST or mutant proteins, their fragments or derivatives. Similarly, co-precipitation studies using antibodies directed against 5'OT EST or mutants thereof, produced as outlined herein, might allow identification of such interacting factors. Such factors, when administered to rats or other animals, or more preferably humans, would be expected to affect the development of obesity and/or male infertility in a similar fashion to that seen in transgenic animals, and would therefore be predicted to have similar uses. The use of transgenic animals or materials derived from them, or from the information disclosed herein, has particular utility in providing a specific means of isolating such factors that interact directly with the novel gene products disclosed herein, as well as in screening their biological activities in vivo.

The invention is described further, for the purpose of illustration only, in the following examples.

MATERIALS AND METHODS

Bacterial cultures.

All media are made with double distilled water and autoclaved prior to use. Liquid cultures of bacteria are incubated with shaking at 37 °C in either LB broth or terrific broth. Bacterial colonies are grown on agar plates made with either LB broth or terrific broth with 15g/l bacto-agar. These media are supplemented with combinations of 20μg/ml or 50μg/ml ampicillin, 20μg/ml tetracycline and 0.2% glucose. Bacterial clones are stored at - 80 °C after the addition of 15% glycerol.

Purification of nucleic acids.

Aqueous solutions containing DNA are purified by vortex mixing with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The emulsion is then centrifuged at 12,000 rpm for 5 minutes in a microfuge at room temperature. DNA is precipitated by adding 3M sodium acetate (pH 5.2) to a final concentration of 300mM and two volumes

of absolute ethanol. The samples are frozen before centrifugation, the supernatant removed and the pellet resuspended in 10mM Tris.HCl pH 8, 1mM EDTA (TE buffer).

DNA preparation from bacteria stocks.

The alkaline lysis method of DNA isolation may be used (Birnboim and Doly, 1979; Sambrook *et al.*, 1989) to prepare plasmid DNA from small volumes of bacterial cultures, typically 10ml. For large scale preparation of plasmid and cosmid DNA, DNA may also be prepared from 1 L overnight cultures by the alkaline lysis method. DNA is dissolved in 100mM Tris.HCl pH 8, 1mM EDTA and further purified on a caesium chloride gradient which is centrifuged at 55,000rpm overnight (Sambrook *et al.*, 1989).

Preparation of genomic DNA from animal tissue.

Rat tail biopsies, up to 1 cm, are taken from 10-14 day old rats and placed in 50mM Tris.HCl pH8, 100mM EDTA, 100mM NaCl ('tail mix'). Genomic DNA is prepared following a standard procedure (Hogan *et al.* 1986) involving incubation with proteinase K, RNase A, phenol extraction and precipitation with isopropanol. Genomic DNA from other tissue such as liver may be prepared by the same method, though this requires additional homogenisation in a larger volume (typically 5ml) of tail mix using a Kinematica Polytron PT 3000 homogeniser prior to the preparation of DNA from a smaller aliquot of homogenate.

Restriction digestion of DNA.

Restriction enzyme digestion is performed using standard procedures in accordance with manufacturers instructions. Enzymes are sourced from Boehringer Mannheim, Cambio, or New England Biolabs. Plasmid DNA is incubated for up to 4 hours whilst genomic DNA digests may be incubated overnight.

Subcloning DNA fragments into plasmid vectors.

Blunting of DNA fragments with a 3' overhang

After digestion of DNA with a restriction enzyme which leaves a 3' overhang, the overhang may be removed by incubation with T4 DNA polymerase to create a blunt end for ligation with other blunt-ended DNA fragments. The digests are phenol-extracted,

ethanol-precipitated with the addition of 10μg tRNA, and resuspended in TE. $MgCl_2$ and deoxynucleotide triphosphates (dNTPs) are added to final concentrations of 10mM and 0.1mM respectively prior to the addition of 2 units of T4 DNA polymerase (New England Biolabs). The reaction is incubated for 15 minutes at 12 °C. The polymerase is inactivated at 75 °C for 10 minutes before purification.

Blunting a DNA fragment by refilling the 5' overhang.

DNA fragments with 5' overhangs may be blunted by filling in the single stranded ends. This may be done using the Klenow fragment of E. coli DNA polymerase I (New England Biolabs). The DNA is digested with an appropriate restriction enzyme, phenol extracted, ethanol precipitated with the addition of 1μg of tRNA and resuspended in TE. 10x Klenow buffer (0.5M Tris.HCl, pH7.6, 0.1M $MgCl_2$) and dNTPs to a final concentration of 1x and 0.2mM respectively are added with 10 units of Klenow fragment. The reaction is incubated at 37 °C for 30 minutes prior to purification of the DNA.

Vector dephosphorylation.

Calf alkaline phosphatase (CAP) may be used to remove 5' phosphate groups from digested vectors to prevent self-ligation during subcloning. Plasmid and cosmid vectors, linearised with restriction enzymes, are incubated with 2 units of CAP (Boehringer) in 50mM Tris.HCl, pH 8.5, 50 mM EDTA, for 30 minutes prior to purification.

Inserting linkers into DNA fragments.

Digested plasmid DNA may be blunted if necessary, phenol-extracted, ethanol-precipitated with the addition of 1μg of tRNA and resuspended in TE. 0.5-1μg of phosphorylated linkers are ligated to linearised, blunt-ended plasmid DNA. Ligations are performed in a final concentration of 1x ligase buffer (50mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25μg/ml bovine serum albumin (BSA), 0.5mM spermidine-HCl with the addition of 400units of T4 DNA ligase (New England Biolabs). The reactions are incubated at room temperature overnight. The enzyme is then inactivated at 65 °C for 15 minutes. The linkered fragments are digested with an excess amount of the appropriate restriction enzyme and the DNA purified prior to further subcloning procedures.

Electrophoresis of DNA fragments

DNA fragments may be electrophoresed in gels of varying percentages of agarose in 90mM Tris-borate, 2mM EDTA, pH 8.0 (TBE buffer) containing 0.5ng/ml ethidium bromide. The DNA bands are visualised on an ultraviolet transilluminator and photographed. Size markers may be Lambda DNA digested with Bst EII, pUC19 DNA digested with Msp I, or a commercially available 1kb ladder (Gibco-BRL).

Purification of DNA fragments.

Digested, blunted, dephosphorylated or linkered DNA fragments are electrophoresed in low melting-point agarose. Gel bands are excised, melted at 65 °C for 5 minutes, and extracted twice with phenol/0.3M NaOAc. Following a phenol extraction and ethanol precipitation with the addition of 1μg tRNA, the DNA is recovered by centrifugation and resuspended in TE. Alternatively, DNA fragments may be purified from agarose using the Prep-A-Gene DNA Purification System (Bio-Rad Laboratories) according to manufacturers instructions.

Purification of larger cosmid-containing fragments.

Large vectors are digested and treated with 50 units of CAP for in excess of 3 hours. EDTA and SDS are added to final concentrations of 5mM and 0.5% respectively. The phosphatase is denatured for 1 hour at 65 °C and the solution is phenol-extracted, ethanol precipitated with the addition of 1μg tRNA, and the DNA recovered is resuspended in TE.

Ligation of DNA fragments into phosphatased vectors.

After purification, DNA fragments and vectors are mixed at equimolar ratios at an approximate concentration of up to 80ng/ml, whilst DNA for recircularisation is used at a concentration of 20ng/ml. Ligation may be done in a volume of 5μl with 200 units of T4 DNA ligase (New England Biolabs) in ligase buffer. Two control reactions are performed simultaneously omitting the insert alone, or omitting both insert and ligase. Ligations are incubated overnight at 16 °C.

Preparation of competent cells.

psi-broth	5g/l bacto-yeast extract, 20g/l bacto-tryptone, 5g/l $MgSO_4$, adjusted to pH 7.6 with NaOH.
TFbI	30mM KAc, 100mM KCl, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% glycerol (v/v), adjusted pH 5.8 with acetic acid and filter sterilised.
TFbII	10mM PIPES, 74mM $CaCl_2$, 10mM KCl, 15% glycerol (v/v), adjusted to pH 6.5 with acetic acid and filter sterilised.

Competent cells yielding a transformation frequency >$5x10^8$ transformed colonies per μg of supercoiled plasmid DNA may be prepared by a method modified from Hanahan *et al.* (1983). Bacteria of the strain DH10B (Grant *et al.*, 1990) are plated on an agar plate and grown overnight at 37 °C. 10ml of psi-broth is then inoculated with 4 colonies from this plate. The bacteria are then shaken at 37 °C until OD550=0.3.

5 ml of this broth is then diluted into 100 ml psi-broth and shaken until OD550=0.28. The flask is then placed on ice, the bacteria are centrifuged at 4 °C for 15 minutes at 2,000 rpm. The supernatant is removed and the pellet allowed to dry briefly before being resuspended in 20ml TFbI. This suspension is left on ice for 5 minutes and then centrifuged at 2,000 rpm for 10 minutes at 4 °C. The supernatant is then removed and the pellet resuspended in 3 ml TFbII and placed on ice for 15 minutes. Aliquots are then frozen on dry ice and stored at -80 °C. The competence of the cells may be tested by transforming plasmid DNA of known concentrations.

Transformation of competent cells.

Competent cells are thawed on ice before 50μl of cells is added to each ligation. These tubes are then incubated on ice for 30 minutes. The cells are then subjected to heatshock at 42 °C for 90 seconds before being placed on ice for 2 minutes. 0.4ml of LB

broth is added and the culture is shaken at 37°C for 1 hour. Cells are then incubated overnight at 37 °C on agar plates containing the appropriate antibiotic. Single colonies are picked with a flamed wire loop and used to inoculate 10ml of media for minipreparation of plasmid DNA.

Packaging of Cosmid DNA into bacteriophage particles.

Cosmid constructs may be packaged into bacteriophage particles using Gigapack II packaging extracts (Stratagene) and E. coli strain DH10B is infected in accordance with manufacturer's instructions.

Southern blotting.

DNA (10µg of genomic DNA or 0.5 µg of plasmid DNA) is digested with appropriate restriction enzymes and electrophoresed on agarose gels with marker DNA of known size. After photography, gels are treated as described by Sambrook *et al.* (1989) and the DNA transferred from the gels onto nitro-cellulose filters by the capillary transfer method (Southern, 1975; Sambrook *et al.*, 1989) and these are then baked for 2 hours.

Radiolabelling of DNA fragments for Southern blots.

DNA probes are obtained by gel purifying appropriate fragments from restriction digests of subcloned DNA. The DNA is denatured by being incubated for 3 minutes in a boiling water bath. The resulting single-stranded DNA fragments are radiolabelled with α-^{32}PdCTP, e.g. by the random primer labelling kit, Prime-It II supplied from Stratagene, in accordance with manufacturer's instructions. The labelling reaction is halted by the addition of TES buffer to a final concentration of 10mM Tris.HCl (pH 7.5), 10mM EDTA, 0.1% SDS. Radiolabelled DNA probes are separated from unincorporated nucleotides by eluting through a column containing Sephadex G-50.

Hybridisation of Southern blots.

100X Denhardts solution	2% BSA, 2% Ficoll 400, 2% Polyvinyl Pyrollidine.

1M sodium phosphate buffer (pH6.6)	352 ml 1M Na_2HPO_4, 648ml 1M NaH_2PO_4.
Prehybridisation mix	0.1mg/ml tRNA, 5x SSC 50mM Na Phosphate buffer (pH 6.6) 10x Denhardts solution, 1% SDS.
Hybridisation mix	Prehybridisation mix with the above described radiolabelled DNA probe.

Filters from Southern blotting are gently shaken at 65 °C in prehybridisation mix for a minimum of 2 hours. This solution is then replaced with hybridisation mix and incubated overnight. The filters are washed in varying concentrations of SSC with 0.1% SDS for varying amounts of time dependent on the DNA probe being used. Filters are then dried and placed between two intensifying screens at -70 °C with Kodak " Xomat-AR" film.

Screening a rat cosmid library

Duplicate filters from a Wistar rat cosmid library containing genomic DNA inserts in the pWE15 cosmid vector (Wahl *et al.* 1987) are prehybridised and hybridised with probes for the rat OT and AVP genes. Following overnight hybridisation, filters are washed with 3x SSC/0.1% SDS for 20 minutes and then SSC/0.1% SDS twice for 20 minutes. Filters are briefly washed in 2x SSC, dried and autoradiographed. Duplicate hybridisation signals are aligned with the master filters and bacteria are picked, placed in media and left to diffuse. The resulting cultures are grown on terrific broth agar with 20μg/ml ampicillin and replica plated. Replica plating of the bacterial culture from the library screening may be performed as previously described (Sambrook *et al.*, 1989). Replica filters are prehybridised and hybridised as above. Positively-hybridising colonies are picked from the master filters and grown in larger volumes of ampicillin-supplemented media for minipreparation and southern blot analysis of the cosmid DNA.

Purification of DNA for microinjection.

50-100μg of DNA is digested with Not I to separate the cVO14 cosmid insert from vector DNA. A salt gradient may be used as described by Dillon *et al.* (1993) to

purify the 44kb fragment. A gradient former is used to pour a gradient ranging in NaCl concentration from 5-25%. The digested DNA is applied to the top of the gradient which is then centrifuged at 5.5 hours at 37,000 rpm. The solution is then removed in 500µl aliquots which are examined by electrophoresis. Fractions containing the fragment to be microinjected are pooled and ethanol precipitated. The pellet is dissolved in microinjection buffer (10mM Tris.HCl, pH 7.5, 0.1mM EDTA). DNA may be purified further using an Elutip column (Schleicher and Schuell) according to manufacturers instructions. cVO14 DNA at a concentration of 1-10ng/µl, typically 2ng/µl, is used to generate transgenic rats.

Superovulation, microinjection and embryo transfers.

40 day old prepubertal female Wistar rats are given intraperitoneal injections of 30 IU pregnant mare's serum (Folligon, Intervet Laboratories Ltd) between 9 and 11 o'clock on day -3. The same rats are injected i.p. at midday on day -1 with 22.5 IU human chorionic gonadotrophin (Chorulon, Intervet Laboratories Ltd) and placed in a cage with a stud male of the same strain. On day 1, females are killed and their oviducts removed and placed in M2 media (Hogan *et al.*, 1986). The oviducts are dissected to release the eggs which are subsequently placed in M2 media with 0.5mg/ml hyaluronidase (Sigma) in order to remove the cumulus cells surrounding the eggs. After 5 minutes the eggs are removed from the hyaluronidase solution, washed thoroughly in M2 and placed in the unbuffered M16 (Hogan *et al.*, 1986) in a 37 °C incubator supplemented with 5% CO_2. After 2 hours of incubation the male pronuclei of the eggs are microinjected using standard procedures and equipment (Hogan *et al.*, 1986). Microinjected eggs are incubated overnight at 37 °C.

The following day (day 2), eggs which have divided to the two-cell stage are washed in M2 media and transferred into the oviducts of pseudo-pregnant adult Wistar rats which have been mated with vasectomized male rats the previous night. The surgery is performed under halothane anaesthetic, with between 15 and 20 eggs being transferred into each infundibulum. Resulting litters are tail-clipped at 2 weeks of age. The tails are used for DNA preparation as described above and analysed by Southern blotting for animals containing transgenes also as described above

RNA preparation.

RNA may be prepared from rat tissue by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski *et al.*, 1987). Briefly, tissue is homogenised in 500μl 4M guanidium thiocyanate, 25mM Sodium citrate (pH 7.0), 0.5% (w/v) sodium N-lauroylsarcosine, 100mM 2-mercaptoethanol prior to the addition of 33μl 3M sodium acetate (pH 4.1), 500μl phenol and 100μl chloroform. The mixture is vortexed and placed on ice for 15 minutes before centrifugation at 12,000rpm for 10 minutes at 4 °C. The aqueous fraction is decanted into a fresh tube and precipitated with isopropanol.

In vitro transcription.

A plasmid containing a T7 polymerase promoter 5' to the inserted sequence is linearised with a restriction enzyme which cuts at the 3' end of the insert. Transcripts are then obtained of the subcloned fragment using a T7 transcription kit (Boehringer Mannheim) according to the manufacturer's instructions

DNA sequencing.

Sequencing of DNA plasmid subclones may be performed manually with the Sequenase version 2.0 sequencing kit (United States Biochemicals) which employs the chain-termination method (Sanger *et al.* 1977), or by automated sequencing using an ABI Prism DNA Sequencing Kit and 377 DNA Sequencer (Perkin Elmer Applied Biosystems) according to manufacturer's instructions.

Reverse Transcription of RNA.

RNA may be converted to cDNA using Superscript II reverse transcriptase (GibcoBRL) according to the manufacturer's instructions, in combination with either an oligo dT primer or another specific primer complementary to the RNA sequence of interest.

Polymerase Chain Reaction amplification of DNA.

The polymerase chain reaction (PCR) may be used to amplify fragments of DNA using 50μl of a reaction mix which contains 10mM Tris, pH8.3, 20mM KCl, 0.2mM

dNTPs, 200nM primers, 50-250ng template DNA, 2.5units Amplitaq DNA polymerase and 1-3mM $MgCl_2$ (the optimal conditions for each amplification are determined empirically). Conditions vary for each template target, but a typical amplification might be to place the reaction mix in a thermal cycler (MJ Research Inc.), denature for 2 minutes and then subject the reaction to 34 cycles of 94 °C for 1 minute, 58 °C for 1 minute and 72 °C for 1-5 minutes, depending on the length of the expected product.

Cloning of PCR products.

Products generated by PCR may be cloned using a TOPO TA Cloning kit (Invitrogen) according to the manufacturer's instructions.

Nuclease Protection assay.

Riboprobe transcripts incorporating ^{32}P may be generated by transcribing approximately 250ng of DNA fragment in Transcription Optimised buffer (Promega), 500mM ATP, GTP and CTP, 20 units Rnasin RNA inhibitor (Promega), 20mM UTP, 100µCi $^{\alpha-32}$PUTP (Amersham) and 20 units of the appropriate RNA polymerase (Promega) at 37 °C for 90 minutes. After treatment of the reaction with 2 units of DNase I (Promega) at 37 °C for 20 minutes, the reaction is denatured and purified by polyacrylamide electrophoresis, followed by excision of the labelled RNA and elution in 350µl of Elution buffer (Ambion) for 2 hours at 37 °C. Nuclease protection may be performed essentially as described by Lee and Costlow (1987) using ^{32}P-labelled riboprobe with 1-20µg total RNA and the RPAII Ribonuclease Protection Kit (Ambion) according to the manufacturer's instructions.

In Situ hybridisation.

Sense and anti-sense riboprobe transcriptsmay be generated using an SP6/T7 transcription kit (Boehringer) with ^{35}S-UTP (NEN Research Products) according to the manufacturer's instructions. *In situ* hybridisations may be performed as described in Bennett *et al.* (1995). Autoradiographs are analysed densitometrically, from a light box using a video camera linked to a Power Macintosh 7600/132 running the programme NIH Image version 1.61.

Immunocytochemistry

Human growth hormone (hGH) may be localised in pituitary and brain sections using a modified avidin-biotin complex immunocytochemistry technique (Bourne *et al.*, 1984). Tissue is collected and fixed in 4% paraformaldehyde for 24 hours. Tissues may be stored at 4 °C in 70% ethanol before embedding in paraffin wax and sectioning. Tissue sections (6μm) are dewaxed in Histoclear (National diagnostics) and rehydrated by sequential 20 sec washes of 100%, 70 % and 30 % ethanol followed by a 1 min wash in distilled H_2O. Endogenous peroxidase activity is inhibited by a 30 min incubation in 3% (v/v) hydrogen peroxidase in methanol. Sections are then washed in distilled H_2O for 1 min before being treated with 0.1% (w/v) trypsin (Sigma) for 15 min at 37 °C followed by 0.5% (v/v) Triton X-100 (Sigma) for 15 mins. After two 5 min washes of distilled H_2O and 0.05M Tris buffered saline (pH 7.6), 0.15M NaCl (TBS) the sections are incubated with 20% (v/v) normal rabbit serum (DAKO) with 5% (w/v) BSA for 30 mins in order to reduce non-specific background staining. The sections are then incubated overnight in a humidity chamber at 4 °C with an antibody specific for hGH, such as sheep anti-hGH primary antibody (1:30,000) (Scottish Antibody Production Unit).

Following two washes in TBS, sections are incubated with biotinylated rabbit anti-goat serum (DAKO, 1:200) for 30 mins. The sections are again washed in TBS and incubated for 30 mins with avidin complexed to biotinylated horse radish peroxidase (DAKO). Human GH immunoreactivity is then visualised by development using 3,3-Diaminobenzidine tetrachloride/hydrogen peroxide (DAB) (4mg/10ml in 0.05M Tris buffer pH 7.6) containing 3% (v/v) H_2O_2. This reaction is quenched in distilled H_2O prior to counterstaining with Gill's haematoxylin (BDH) and coverslipped for microscopic examination.

Radioimmunoassays

Tissue samples are homogenised in varying volumes of phosphate buffered saline with either glass homogenisers (for volumes up to 1ml) or a Kinematica Polytron PT 3000 homogeniser (for larger volumes). The same polyclonal sheep anti-hGH antibody (Scottish Antibody Production Unit) may be used to measure hGH in tissue extracts by RIA as described in Fairhall *et al.* (1992) using recombinant hGH as standard. AVP may

be measured by RIA as described in Horn, Robinson & Fink (1985). Rat GH may be measured as in Charton *et al.*, (1988). Bovine neurophysin may be measured by RIA using a specific antiserum that does not recognise rat neurophysins (Gordon Weeks, 1987). Rat leptin and rat insulin may be measured by specific RIAs using kits from Linco Research Inc, following the manufacturer's instructions. Corticosterone may be measured by a double antibody RIA kit obtained from ICN Biomedicals. Cholesterol and triglycerides in blood samples may be measured using kits obtained from Sigma Diagnostics ('Cholesterol 20' and 'Triglycerides, UV'). Plasma glucose values may be measured using a Beckman glucose analyser.

EXAMPLE 1

ISOLATION OF COSMID DNA, CONSTRUCTION OF TRANSGENE COSMIDS AND GENERATION OF TRANSGENIC RATS

Isolation of cosmid DNA

Since the DNA sequences of the rat AVP and OT genes and their orientation and structural relationship to each other in the rat genome are known (Ivell & Richter, 1984a; Mohr *et al.* 1988; Schmitz *et al.*, 1991) the size of restriction fragments which should be detected with cDNA probes for these genes can be predicted. Colonies bearing rat DNA which contained fragments hybridising to these OT and AVP probes in the same areas in duplicate filters from the cosmid screening are aligned with the original bacterial plates. These colonies are picked and grown and DNA prepared from them, digested with Hind III, run on agarose gels, Southern blotted and hybridised to the same OT and AVP probes again. Three positive colonies are chosen for further analysis because their differing restriction fragment patterns indicated that they spanned different regions of the rat AVP/OT locus. From Southern blotting of restriction digests using probes against the first exon of each gene and the vector, they are found to span a total of 44kb, including both genes, the 11kb intergenic region, 8kb of AVP 5' flanking sequence and 24kb of OT 5' flanking sequence. These three overlapping cosmids are designated cVO1, 2 and 3. An overall schematic map of this region, indicating the location and orientation of AVP and OT genes, the location of some important restrictions sites, and areas of sequence known or subsequently determined and disclosed here, is shown in Figure 1

To facilitate further restriction mapping of the 5' flanking sequence of the rat OT gene, 8kb and 14kb Sma I fragments and an 8.5kb Kpn I fragment are subcloned into cloning vectors and subjected to further restriction mapping. Smaller fragments of the OT and AVP genes are also subcloned into pUC 19 derived plasmid vectors and used to remove restriction enzyme sites and to insert the reporter genes into the rat OT and rat AVP loci. Oligonucleotide linkers containing sequences for unique restriction sites are also inserted in the 5' untranslated regions of the two genes to allow for future modifications of this construct.

The subcloning strategy used for the AVP locus is outlined in Figure 2 Essentially, the aim is to insert a genomic hGH reporter fragment in a unique cloning site introduced into the 5' untranslated region of rat AVP gene. Swanson *et al.* (1985) had previously shown that hGH reporter transcripts, when fortuitously expressed, may be expressed and translated efficiently in these neuronal cells types. Furthermore, hGH nucleotide sequences can be differentiated from rat GH sequences by specific nucleotide probes (Seeburg *et al.*, 1977; Roksam & Rougeon, 1979) and the protein can be differentiated from rat GH by specific antisera (Appendix 1). An Mlu I linker is initially inserted into a smaller subclone of the rat AVP gene, replacing the Dra III site in the 5' untranslated region. A genomic fragment of the human GH structural gene (Roksam & Rougeon, 1979) is then inserted as an Mlu I-linkered fragment spanning from the 5' untranslated region of the hGH gene to a region 3' of the last exon and containing all 5 exons and 4 introns of hGH. This AVP-hGH fragment is inserted as a 12.2 kb Cla I-Xho I fragment containing 450bp 5' and 8kb 3' of the transgene, with deletion of other Xho I restriction sites.

The subcloning strategy used for the OT locus is outlined in Figure 3. In this case the aim is to replace most of the rat OT structural gene with corresponding bovine sequences (Land *et al.*, 1983). The protein produced should function identically, but the bovine sequences would provide a 'silent' reporter since they could be differentiated from rat sequences (Mohr *et al.*, 1988) by specific nucleotide probes, and the protein differentiated from rat neurophysin by specific antisera. Rat neurophysin has previously been used as a

transgene reporter in mice (Belenky *et al.*, 1992). Due to the constraints of suitable restriction sites it is necessary to assemble a 5' construct of the hybrid rat/bovine OT gene (containing exon A and most of exon B) and a 3' construct (containing a small fragment of exon B and exon C) separately. These constructs are joined to produce the hybrid gene with the 5' and 3' flanking sequences being added in subsequent steps. A Sal I linker is also inserted immediately 5' to the translational start site of the bovine OT to provide a unique cloning site within the AVP/OT locus for future modification of the construct. The hybrid gene is inserted into the final construct as a 10.5 kb Mun I - Xho I fragment containing 7.8 kb of 5' and 1.7 kb of 3' flanking sequence, after deleting other restriction sites within the cosmid

Assembly of the final construct.

The pWE15 vector is modified to remove the unrequired SV2 neomycin gene. This reduced the vector size from 8.5kb to 4.2 kb and therefore increased the size of the insert that could be subcloned into the cosmids, which can efficiently package up to 52 kb (Wahl *et al.* 1987). Cla I, Mun I, Sal I and Mlu I restriction sites are also removed from the vector to permit subsequent cloning steps. The pWE15 cosmid vector has Not I sites flanking the insert. Restriction mapping of cVO1 revealed a Not I restriction site 13kb upstream from the rat OT gene (Figure 1) which would also be digested if Not I is used to remove the insert. Therefore, a 4.6kb Aat II-Sca I fragment containing this site is subcloned, the Not I site deleted, and the fragments ligated and replaced into the construct. Digestion of the ligation product with Not I confirmed that this site had been destroyed.

The modified OT and AVP gene fragments are inserted into cVO3, followed by addition of the 5' region present in cVO1 but not in cVO3using an Aat II fragment. This adds all except 1.1kb of the extreme 5' end of cVO1 and contains a Not I linker at the extreme 5' end.

The final construct, termed cVO14, spans 44kb and includes 8kb 5' of rat AVP, 24kb 5' of rat OT and 11kb of intergenic sequence. The construct has reporter gene hGH sequences inserted into the 5' untranslated region of the rat AVP gene and parts of the

bovine OT gene sequences substituted for equivalent rat OT gene sequences. The final cVO14 construct is illustrated diagrammatically in Figure 4.

Generation of transgenic rats bearing the cVO14 construct.

The 44 kb Not I insert is released from cVO14 by Not I digestion, purified on a salt gradient and microinjected into fertilised rat oocytes. These embryos are transferred into pseudopregnant mothers and the offspring are analysed for the presence of the transgenes. Genomic DNA prepared from tail biopsies of these pups is digested with Bgl II, Southern blotted and hybridised with a radiolabelled genomic hGH probe that should identify 2 predicted fragments of 0.9 and 2.1 kB from transgene DNA. This probe does not detect endogenous rat GH sequences. Of 102 pups the hGH transgene is present in the DNA of only 3 pups, termed JP 17, JP 19 and JP 59. JP 19 dies at 11 days of age, and is not analysed further.

Other samples of DNA from the two remaining rats is digested with Pst I, Southern blotted and hybridised with a radiolabelled probe that should identify two predicted fragments of 0.9 and 1.6kB from the hybrid rat/bovine transgene sequence, and a single 2.5kB fragment from the endogenous OT gene. Both JP17 and JP59 rats are also found to contain this hybrid gene, as well as the endogenous gene, whilst only the endogenous fragment is visualised in DNA from non-transgenic rats.

DNA from JP 17 and JP 59 rats is also Southern blotted and probed with radiolabelled DNA fragments corresponding to the ends of the cVO14 construct, which confirmed that whole copies of the microinjected fragment are present in both rats. The copy number of the transgenes is estimated by Southern blotting of Hind III fragments and hybridisation with a probe for the rat AVP gene sequences, which recognised a 3.4 kb fragment corresponding to the endogenous rat AVP gene and a 5.2 kb fragment which represents the transgene with its hGH reporter gene insertion. Assuming equal affinity to endogenous or transgene sequences, phosphorimaging these blots suggested that the JP17 rats contained at least 4 copies of cVO14 whereas JP59 rats had a single copy. The copy

number and restriction pattern of the transgenes remained consistent through successive generations of breeding, suggestive of a single site of chromosomal integration.

Further analysis of DNA suggested that the insert contained concatamers of cVO14 in JP17 but not in JP59, and that one concatamer pair contained a truncation which removes a fragment of approximately 1kb between 8kb and 7kb 5' of rat AVP. Restriction mapping and sequence analysis of the cosmid ends enabled us to design PCR primers (PL216 (SEQ. ID. No. 10 and PL210 (SEQ. ID. No. 9)) that uniquely identify DNA from JP17 rats bearing this insert, and distinguishes them both from non-transgenic littermates, and from JP59 rats bearing a single copy of this insert.

Establishing colonies of transgenic rats.

The founder JP17 rat is a male. He sires only single litter of rats at 6 months of age although constantly caged with fertile females. This litter contains both male and female rats bearing the transgene, indicating that the integration has occurred onto an autosomal chromosome. No further litters are sired by male progeny. Litters bred from transgenic JP17 female progeny show an approximate 1:1 ratio of transgenic to non-transgenic rats (46 transgenic versus 54 non-transgenic in the first 100 pups) suggesting that the transgene does not have a detrimental effect on embryonic viability.

The founder of the JP 59 line of rats is female and bred normally (the ratio of transgenic to non-transgenic pups is approximately 1:1 (47 transgenic verses 53 non-transgenic in the first 100 pups). This single copy integrant is also present on an autosomal chromosome since male JP59 rats of this line sire transgenic progeny of both sexes.

EXAMPLE 2

ANALYSIS OF EXPRESSION OF EXPECTED TRANSGENE PRODUCTS

Human growth hormone

The expression of hGH from cVO14 is investigated in both JP59 and JP17 rats. Immunocytochemistry shows expression of hGH protein in hypothalamic magnocellular paraventricular (PVN) and supraoptic nuclei (SON). Human GH immunoreactivity is also

transported via axons passing through the internal zone of the median eminence and present in the axon terminals in the posterior pituitary.

In situ hybridisation confirms strong expression of hGH in the PVN and SON of transgenic rats of both JP59 and JP17 lines. hGH transcripts are also detected in other sites of AVP expression in the CNS in JP 17 transgenic rats, such as the medial amygdaloid nucleus and the habenula (Buijs, 1987; Caffe *et al.*, 1987; Urban *et al.*, 1990). Double *in situ* hybridisation analysis or immunocytochemical analysis confirms that hGH expression is localised in AVP neurones, and not in OT neurones. In independent studies, RT-PCR analysis detects hGH transcripts in hypothalami and pituitaries from both lines, and also detected transcripts in the pancreas and also faintly, in adrenals of JP 17 rats, but not in other tissues tested. These findings are in accordance with previous reports of extrapituitary expression of the endogenous AVP gene in these tissues.

Radioimmunoassay for hGH confirms the presence of significant quantities of hGH in posterior pituitary extracts from both JP59 and JP17 animals, with larger amounts in JP17 line consistent with their higher copy number. Small amounts of hGH immunoreactivity are also found in the pancreas (0.016 ± 0.0075 ng/mg of tissue, n=3) of 20 week old JP 17 male rats, though this represents < 0.1% of the amounts of hGH found in the posterior pituitary extracts of the same rats (168ng $\pm$ 16 ng/mg, n=5). Thymus, heart, kidney, fat, liver, ovary, uterus, testis, lung, cortex, cerebellum, spleen and adrenals all had undetectable levels of this protein (<0.0004ng of hGH/mg of tissue).

If the hGH transgene is correctly expressed, then stimuli for increased AVP synthesis and release should increase hypothalamic expression of the hGH transgene and decrease pituitary stores of hGH. Chronic osmotic stimulation has been shown to regulate the expression of the AVP gene (Lightman *et al.*, 1987; Murphy *et al.*, 1990) and cause a release of AVP from the posterior pituitary (Fitzsimmons *et al.*, 1994). The stimulus of salt-loading has previously been used to detect whether the DNA regulatory regions responsible for physiological regulation of the rat AVP gene are present within microinjected constructs (Zeng *et al.*, 1994b; Waller *et al.*, 1996). Groups of non-transgenic or transgenic JP59 or JP17 male rats given 2% NaCl w/v in their drinking

water for 72 hours both show a marked increase in hGH expression in PVN and SON. Furthermore, posterior pituitary hGH content fall significantly in such salt-loaded animals in parallel with the fall in AVP content. Samples taken from JP17 rats confirm that hGH is secreted, and can be detected in plasma by RIA (1.3 ± 0.09 ng/ml)

The effects of transgenic expression of hGH to reduce rat GH by feedback have been documented earlier in other transgenic rats (Flavell *et al.*, 1996) Therefore, rat GH content of the pituitaries of JP 17 rats and non-transgenic littermates are measured by RIA. Rat GH is significantly reduced in both the male and female JP 17 transgenics in comparison to the non-transgenic controls at 23, 77 and 140 days of age. At 140 days, the mean pituitary rat GH content of male JP 17 rats is 34.2% of that of the age-matched non-transgenics. The pituitary rat GH content is less affected in the female JP 17 rats (57.4% of the mean rat GH content of the non-transgenics, $p<0.02$).

The size of the anterior pituitaries also suggests that there is a reduction in their cell number as JP 17 male rats at 140 days have significantly smaller anterior pituitaries than non-transgenic controls (4.6 ± 0.1mg for JP 17 males versus 8.2 ± 0.5mg for wild-type rats, $p<0.002$, n=6). The pituitaries of JP 59 males and females do not show a reduction in rat GH content or size ($p>0.55$).

Bovine neurophysin

No bovine OT-NP protein can be detected in posterior pituitary extracts (<10pg per pituitary) from JP17 rats, using a specific RIA that distinguishes bovine neurophysin from rat neurophysins (Gordon-Weeks, 1987). An RT-PCR assay for bovine OT-NP transcripts is applied to hypothalamic extracts of adult males or of lactating female rats culled within 24 hours of littering, from both lines. The latter animals are chosen since they should show higher levels of endogenous OT expression (Van Tol *et al.*, 1988). PCR is performed on cDNA generated by reverse transcribing RNA from various tissues of both JP 17 and JP 59 lines (hypothalamus, pituitary, pancreas, ovary, heart, lung, muscle, thymus, cerebellum, uterus, testis, spleen, kidney, adrenals, liver, cortex). Additional reactions with primers for ß-actin and hGH are also included. The reactions of the rat/bovine OT primers with the cVO14 construct and in vitro transcribed rat/bovine

OT RNA both yielded the correct size fragment (767bp), but no transcripts from the rat/bovine OT transgene are detected in any tissue. We conclude that the rat/bovine OT portion of the cVO14 construct is not detectably expressed in JP 17 or JP 59 transgenic animals.

The lack of expression of the rat/bovine OT transgene may indicate that additional sequences lacking from cVO14 are required to achieve appropriate OT expression in addition to expression from the AVP locus. Other possibilities are that alterations introduced into the OT locus prevent expression. This could be in the coding regions of the hybrid rat/bovine OT cassette. Another possibility is the introduction of the Sal I linker 5' of the OT gene. A further possibility is the presence of a base change in the region immediately 3' of the TATA-box which is discovered upon sequencing this region of cVO14 (Figure 5).

EXAMPLE 3
DISCOVERY AND ANALYSIS OF *5'OT-EST* AND *5'OT-EST -XDEL*

cVO14 is noted to contain a CpG island 13kB upstream of OT. Sequencing of 3.3kB of this region of the cosmid reveals a potential novel gene. Comparison with EST sequences in the public databases revealed partial matches to sequences from rat, human and mouse origin. The GenBank accession numbers for such ESTs include: H31114; H31115; AA955566; AA850004; AA104183; AA080247; AA245389; AA242211; AA421310; AA505752; AA421393. Such searches also reveal a partial match to a human genomic sequence GenBank Accession number AF036329. From comparisons with these sequences and the rat genomic DNA sequence disclosed herein, it is predicted that the novel rat gene in cVO14 contains four open reading frames, termed w, x, y, z. This gene is termed *5'OT-EST*. The genomic DNA sequence and predicted exon structure is disclosed in SEQ. ID. No. 16. The gene predicts an open reading frame (SEQ. ID. No. 1) and a protein of 200 amino acids, termed 5'OT-EST, whose structure is disclosed in SEQ. ID. No. 2. Comparisons with EST sequences from human and mouse sources and alignment with full length sequences from rat DNA enable the prediction of homologous cDNA and protein sequences in these species and these are disclosed in SEQ. ID. No. 3

and 4 and SEQ. ID. No. 5 and 6 respectively. The protein sequences predicted from these predicted cDNAs are highly homologous, as shown in Figure 6.

A Not I restriction site is identified approximately 13kB upstream of OT in cVO3. As described in Appendix 2, this site is deliberately destroyed during the construction and assembly of cVO14 in the pWE15 cosmid vector, as the construct required Not I sites only at the ends of the insert. However, sequence analysis of cVO14 reveals that the Not-1 site lies in *5'OT-EST*, more precisely, in exon w of *5'OT-EST*. Furthermore, this sequence analysis reveals that in addition to destroying the Not I site, the procedure used (digestion, filling in and religation) also resulted in an additional unpredicted deletion of 412bp. This deletion includes all of the sequences recognised as exon x as defined herein. The mutated form of this gene, lacking sequences including those for exon x, is therefore termed herein *5'OT-EST-xdel* for the purposes of this application. Its sequence, and the structures of the predicted exons from this form of the gene are disclosed in SEQ. ID. Nos. 7 and 8. The presence of *5'OT-EST-xdel* in the genome of JP17 and JP59 rats is confirmed by the generation of the predicted shorter product upon amplification of genomic DNA from these animals by PCR with primers PL266 (5'TCATGTTGCGGGCTTTGAAC) and PL271 (5'TCTTTCAGTTGCACCCAAGC) which flank the deletion (see SEQ. ID. Nos. 11 and 12 respectively).

The form of *5'OT-EST* that is incorporated in both JP17 and JP59 in 4 or 1 copies, respectively, is mutated from the wild type sequence. *5'OT-EST-xdel* would be predicted to give rise to an altered mRNA, which if translated would produce a truncated protein product with an additional novel amino acid sequence. The predicted sequence of this novel product, termed herein 5'OT-EST-xdel is disclosed in SEQ. ID. No. 8. Comparison with the aligned predicted protein sequences of 5'OT-EST in normal rats and in other species predicts that the protein translated from this RNA would contain an altered exon w, with a novel C-terminal peptide sequence (shown in lower case beginning at the arrow in Figure 6) predicted to arise by translation of DNA sequences normally present as part of an intron in *5'OT-EST*. Searches in the protein databases in the public domain do not find any significant matches of this mutated protein sequence to known sequences.

To demonstrate that both the endogenous and truncated forms of *5'OT-EST* are transcribed in JP59 and JP17 rats, PCR primers are designed which can distinguish between these gene products. The sequence of these primers is given in SEQ. ID. No. 11, 12 and 13. RT-PCR using these primers confirms the presence of transcripts from the endogenous form of *5'OT-EST* in testicular RNA extracts from JP17, JP59 and wild-type rats, but the presence of a transcript with the 412bp deletion only in such tissue extracts from JP17 and JP59 rats. Sequencing of amplification products generated by PCR with primers PL266 (SEQ. ID. No. 11) and PL273 (SEQ. ID. No. 13) from wild type and JP17 rats confirms this region of the sequence of the endogenous rat transcript as well as the truncated 5'OT-EST-xdel sequence disclosed in SEQ. ID. No. 8. Extracts of a rat adrenal medullary cell line (PC12 cells) also contain an RNA product of *5'OT-EST* of the expected size.

Identification and sequencing of *5'OT-EST* and *5'OT-EST-xdel* enables the design of probes to carry out *in situ* hybridisation and RNAse protection analysis for the products of these genes on normal and JP17 rat tissue extracts. *In situ* hybridisation with probes complementary to exons w or z (more specifically, corresponding to bases 1020-1167 and 2229-2451 of Fig 4.1 respectively) on hypothalamic sections from wild-type or transgenic animals, revealed a highly specific expression in magnocellular SON and PVN. No other specific expression in different brain regions is observed at this level of detection. This is an unexpected finding, which is repeatedly confirmed. *5'OT-EST* is a novel member of the AVP/OT locus and is expressed in the same hypothalamic magnocellular neurones. Similar patterns of expression are seen with both probes and no differences in tissue distribution of hybridisation signal are seen between wild-type or JP17 tissues. Further *in situ* hybridisation analysis on a wide variety of tissues reveals strong expression in the testis consistent in distribution with tubular or Sertoli cell expression. Sparse expression is also seen in other tissues, including lung, spleen, intestinal smooth muscle and adrenal gland.

From the sequence information, it is further possible to design probes for *in situ* hybridisation analysis that distinguish completely between the forms of mRNA produced from *5'OT-EST* and *5'OT-EST -xdel.* More specifically, oligonucleotide probes directed

against transcripts containing exon x would be predicted to detect *5'OT-EST* but not *5'OT-EST -xdel* transcripts, whilst probes directed against the intron sequence in *5'OT-EST* that immediately follows the truncation in *5'OT-EST -xdel* detect transcripts containing this sequence, that code for the truncated product in extracts from rats expressing *5'OT-EST -xdel* transcripts (such as JP17 and JP59 rats) but not from non-transgenic rats. Examples of such probes are given in SEQ. ID. Nos. 14 and 15.

An oligonucleotide probe of the sequence depicted in SEQ. ID. No. 15 (specific for the truncated sequence) is used for in situ hybridization and confirms transgene expression specifically in PVN and SON in JP17 rats, whereas no signal is observed in PVN or SON sections from non-transgenic rats, hybridized at the same time with this probe.

Nuclease protection analysis may also be performed using a riboprobe to exon w as described above. From the sequence we disclose herein, this probe would be predicted to protect 147bp and 94bp bands from transcripts from *5'OT-EST* and *5'OT-EST - xdel* respectively. Using such a probe to analyse testicular RNA extracts confirmed that the full length transcript is present in both transgenic and non-transgenic animals and that the truncated product is present in JP17 and JP59 extracts in a level consistent with the copy number of the cVO14 insertion, that the truncated transcript is indeed absent from non-transgenic testis extracts. The full length product is present in control extracts of PC12 cells.

The gene termed *5'OT-EST -xdel* present in cVO14 in both JP59 and JP17 rats is transcribed in several tissues in JP17 rats, specifically in hypothalamic cells and in testicular cells, and that the sequence of the truncated transcripts if translated, would give rise to a protein product that is severely truncated with respect to the normal gene product and would an contain additional novel peptide sequence.

EXAMPLE 4

PHENOTYPIC ANALYSIS OF JP17 TRANSGENIC (SLOB) RATS

Growth measurements

JP 17 transgenic rats of both sexes and non-transgenic littermates are weighed at regular intervals. Male JP 17 rats show a slight but significant reduction in their body weight up to 120 days of age ($p<0.01$). This juvenile growth retardation is not seen in females of this line or the rats of either sex of the JP 59 line whose body weights are not significantly different to those of the non-transgenic groups ($p>0.7$). This effect disappears with time. At 140 days, the weight difference between JP 17 and non-transgenic male rats is no longer significant. Some organs of 140 day old rats are dissected and weighed. Heart weights do not differ significantly ($p<0.14$) but the weights of the kidneys (0.99 ± 0.03g in JP 17 rats versus 1.24 ± 0.05g in non-transgenic rats, $p<0.001$), liver (11.11 ± 0.29g in JP 17 rats versus 14.40 ± 0.32g in non-transgenic rats, $p<0.001$) and spleen (0.66 ± 0.02g in JP 17 rats versus 0.84 ± 0.03g in non-transgenic rats, $p<0.001$) differs in weight (n=6 in all groups). Disproportionate growth is well known in transgenic animals expressing hGH (e.g. Shea *et al.*, 1987)

Body weight measurements in ageing JP 17 rats.

After about 140 days, the group of JP 17 transgenic male rats gain weight more rapidly than their non-transgenic littermates (Δ weight between 200 and 420 days 356.5 ± 57.419g for JP 17 males versus 182.50 ± 7.554 for non-transgenic males, $p<0.03$, n=5, Figure 5.1). Female JP 17 transgenic rats show only a slight increase in weight gain when compared to non-transgenic littermates (Δ weight between 280 and 480 days 111.8 ± 8.2g for JP 17 females versus 88 ± 5.1g for non-transgenic females, $p<0.04$, n=6). This is illustrated in Figure 8, which clearly shows the sexually dimorphic weight gains in these animals. No significant increased weight gain is observed in either sex of transgenic JP 59 rats compared with non-transgenic JP59 rats. At one year, the weights of the kidneys and liver of JP 17 male rats have reached a value that is not significantly different than that of the non-transgenic rats (n=6 in both groups) ($p<0.08$ for kidneys and livers), but the spleens remain lighter (1.03 ± 0.04g versus 1.225 ± 0.05g in wild-type rats, $p<0.01$).

These organs in transgenic JP 59 rats show no variation from their non-transgenic littermate controls (p>0.43).

Body length, width and fat-pad measurements.

Measurements of the body lengths (nose-anus) and the width across the pelvic area of anaesthetised male JP17 and non-transgenic rats are taken (Figure 9). At 20 weeks of age male JP 17 transgenic rats are shorter than their littermate non-transgenic controls with an increased width across the pelvic area. At 52 weeks, the difference in nose-anus length is no longer significant but the girth of the transgenic JP17 rats has increased greatly whereas the non-transgenic rats only exhibit a moderate increase in girth. This late-onset increase in the body weight/length ratio is shown in Figure 10.

A comparison of the body proportions of a live SLOB rats and non-transgenic littermates shows a marked increase in abdominal fat. This is also obvious when individual peri-renal fat pads are compared. To evaluate this abdominal distribution of extra fat in SLOB rats, peri-renal and testicular fat pads are dissected and weighed from matched groups of male JP 17 and non-transgenic littermates of 77, 140 and 365 days of age, and the results are shown in Figure 11. The peri-renal fat pads of the transgenic rats are markedly increased in weight at both 140 days and 365 days when their mean weight is almost five times that of the non-transgenic animals. The testicular fat, however, did not show a comparable increase. Although testicular fat pad weights are marginally larger than those of the non-transgenic rats at 140 days (p<0.05), no further significant increase occurs during the period of a large accretion in peri-renal fat, and there is no difference in testicular fat pad weights at 365 days between SLOB rats and their non-transgenic littermates, despite their much larger body weight, and evident gross visceral obesity.

In other matched groups of 1 year old SLOB rats and non-transgenic littermates of both sexes, plasma cholesterol, triglycerides, glucose, insulin, leptin and corticosterone are measured in blood samples taken when the animals are killed, and the results are summarised in Figure 12. Plasma triglycerides are modestly but significantly elevated in SLOB males compared to non-transgenic males. There are no differences in plasma triglycerides in females. Cholesterol levels are no different between the groups. Plasma

glucose and insulin values are also in the normal range and did not differ between the groups, suggesting that the obesity is not secondary to diabetes or insulin resistance. Plasma corticosterone is also in the normal range in all groups of rats. Notably however, the plasma leptin levels are elevated significantly in both male and female SLOB rats compared with their non-transgenic littermates, and are almost two-fold higher in SLOB males than in SLOB females. Leptin receptor transcript isoforms are also expressed in normal amounts in the hypothalamus, piriform cortex and choroid plexus. These increases would be expected given their increased body fat, but prompted a study of their food intake.

A further group of 5, 11-month old SLOB rats and 5 non-transgenic rats are housed singly in metabolic cages for 14 days, and after a period of acclimatisation to single housing, food intake is measured over the last four days of the experiment. There is no significant difference in food consumption between the two groups (SLOB rats 23.4 ± 1.1g/day vs. 23.5 ± 1.8 g/day in the non-transgenic males, Mean ± S.D.).

Although the SLOB phenotype, as demonstrated by the forgoing, has a striking late-onset feature, the phenotype is latent at a younger age and can be induced by increasing the levels of fat in the diet. This is demonstrated by observing the phenotypic differences resulting from feeding two groups of 100 day old transgenic and normal littermates either regular rat chow, which has a fat content of 4%, or a high fat diet having a fat content of 30% over a 27 day period.

The rats fed a normal diet show no significant difference in weight gain between transgenic and non-transgenic littermates. However, in the case of the rats fed on a 30% fat diet, the transgenic animals gain twice the weight of their non-transgenic littermates (see Figure 13). Controls in dwarf rats show that the obese phenotype is not due to growth hormone deficiency.

Plasma leptin levels are measured at sacrifice. These are found to be higher in transgenic animals, and rise in both transgenic and non-transgenic rats fed on a 30% fat diet.

Moreover, the increase in dietary fat is associated with a significantly reduced food intake in normal rats, but not in SLOB rats, despite their higher leptin levels.

Induction of obesity in ovariectomised female rats.

Four groups of female rats are studied (see Figure 14). Sham-operated transgenic female SLOB rats are lighter than non-transgenic sham-operated female littermates at 100 days, but gain the same amount of weight in the following 11 week period (Δwt 45.5 ± 5.3g, vs Δwt 48.4 ± 3.8g). In rats ovariectomised under anaesthesia, both groups show an increase in weight gain; this increase is much higher in SLOB rats than in non-transgenic littermates (Δwt 128 ± 7.7g vs 89 ± 4.3g, $P<0.001$). Some animals from each group are killed 18 weeks post ovariectomy and their supra-renal fat pads dissected and weighed. The fat pad weight is much larger after ovariectomy in SLOB rats (4.67 ± 0.61vs 1.37 ± 0.39g in ovariectomised versus sham-ovariectomised SLOB females, $P<0.01$), than in nontransgenic rats (2.33 ± 0.94g vs 1.0 ± 0.01g in ovariectomised vs sham-ovariectomised nontransgenic littermates).

Fertility of the JP 17 male rats.

Twelve JP 17 and twelve non-transgenic young adult males are each housed with two 12-week old normal females, for several consecutive days. The female rats are examined every morning for evidence of copulation, either in the form of a vaginal plug or sperm in vaginal smears, and are observed for a sufficient amount of time to allow any litters conceived during this time to be born. No litters are sired in this time by JP 17 males, whereas 11 of the 12 females housed with wild-type males produced litters.

The immediate cause of infertility in male SLOB rats is unknown. The size and gross anatomy of their testes and seminal vesicles is normal, suggesting unaffected levels of gonadotrophins or androgens. Testicular size, sperm morphology, motility and testosterone levels all appear normal in SLOB rats. Treatment of a SLOB male rat with exogenous androgens did not improve fertility. One cause could be hGH, since infertility is a common problem in GH transgenic animals (Yun *et al.*, 1987, Bartke *et al.*, 1988, Flavell *et al.*, 1996) and male transgenic animals expressing hGH have been reported to have a reduced frequency in the impregnation of females (Bartke *et al.*, 1992). However

female SLOB rats also express equivalent amounts of hGH and are not infertile. Furthermore, we found no evidence for hGH expression or of hGH protein in testes from SLOB rats.

In contrast, the expression of *5'OT-EST* in normal rats and the high level of a truncated RNA product from *5'OT-EST -xdel* in hypothalamus and in particular, the testis from SLOB male rats, and the lack of expression of either product in ovaries in SLOB females, leads us to conclude that the novel infertility and obesity phenotype more probably results from the presence of multiple copies of *5'OT-EST -xdel* in SLOB rats. A disruption of testicular function by *5'OT-EST -xdel* and consequent infertility is part of, may partly contribute to, or exacerbate the degree of the male-preponderance of, the obesity phenotype of SLOB rats. A testicular disruption is not absolutely required however, since a mild visceral obesity can also be discerned in SLOB females.

Longevity of SLOB male rats.

The longevity of JP 17 also appears to differ to that of normal rats. Six male JP 17 rats and six wild-type rats are housed under constant conditions. After two years, all six JP 17 rats have died, five at between 10 and 14 months of age and the sixth at 21 months of age, whereas only a single wild-type rat has died at 13 months. The longevity of JP 17 females or JP 59 males or females has not been similarly investigated.

Comparison of phenotype with other rat obesity models

When comparing the phenotype in SLOB rats with other findings reported in the literature, the closest parallels are lines of transgenic rats expressing hGH driven by a mouse whey acidic protein promoter, (Ninomiya *et al.*, 1994; Ikedae *et al.*, 1994,1995, 1997). Ikeda *et al.* (1995) described two lines of rats expressing high or low hGH levels in serum. Gigantism is observed in the high hGH-expressing line, but visceral obesity is also observed in the low-expressing line, associated with endogenous GH suppression. No sexual dimorphism is reported, and the obesity is associated with carbohydrate metabolic disorders, hypertriglyceridaemia and insulin resistance. Ikeda *et al.* 1995 specifically concluded that the effect is due to differences in serum hGH levels affecting carbohydrate metabolism. A later study in these rats (Ikeda *et al.* 1997) reported female

infertility and enlarged ovaries which further distinguishes this phenotype from that seen in SLOB rats.

In common with the rats reported by Ikeda *et al.* (1994), SLOB rats also show reduced rat GH production and secretion. GH deficiency is associated with increased visceral fat in humans, but this can be alleviated by hGH treatment. However, isolated rat GH deficiency is an unlikely cause of obesity in SLOB rats as other lines of severely GH deficient dwarf rats (Charlton *et al.*, 1988) do not develop obesity when housed under identical conditions to SLOB rats. Obesity can be induced in such dwarf rats (as in normal rats), when placed on high fat diets for prolonged periods though females are more susceptible than males (Clark *et al.*, 1996). A similar pituitary GH suppression is also evident in female SLOB rats but they do not develop the same massive abdominal obesity as males. Pituitary rat GH suppression is also seen in the non-obese JP59 rats of both sexes and in Tgr rats (Flavell *et al.*, 1996) which do not develop obesity.

The defects in other genetic models of obesity in the rat have recently clarified; examples of these include the Zucker fa/fa rat, the Koletsky (f) obese rat, the JLA/cp corpulent rat, and the OLETF rat, and their related sub strains (Iida *et al.*, 1996; Wu-peng *et al.*, 1997; Takaya *et al.*, 1996; Lee *et al.*, 1997; Kahle *et al.*, 1997;. None of these show the male specificity, late onset or pattern of distribution of obesity seen in SLOB rats and they exhibit significant hyperglycaemia and insulin resistance, which again distinguishes them from SLOB rats. Male specificity, infertility, extremely late onset of obesity, a highly selective visceral accumulation of fat, but relatively normal metabolic profile, without insulin resistance, hyperphagia or hyperglycaemia distinguishes the dominant phenotype in the SLOB rats from all other known models of obesity in the rat, including those with low endogenous rat GH expression or hGH expression from other transgenes.

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Claims

1. 5'OT-EST polypeptide having a sequence selected from the group comprising the sequences set forth in any one of SEQ. ID. Nos. 2, 4 or 6, and sequences substantially homologous to any one of the polypeptides set forth in SEQ. ID. Nos. 2, 4 or 6.

2. A polypeptide according to claim 1 comprising an amino acid sequence encoded by at least one exon selected from the group consisting of exons w, x, y and z as set forth in SEQ. ID. No. 16, or equivalents thereof as set forth in any one of SEQ. ID. Nos. 3 or 5.

3. A polypeptide according to claim 2, which comprises an amino acid sequence encoded by at least part of exon w as set forth in SEQ. ID. No. 16, or equivalents thereof as set forth in any one of SEQ. ID. Nos. 3 or 5.

4. A mutant of a 5'OT-EST polypeptide according to any preceding claim which is capable, *in vivo*, of modulating the obesity of an animal expressing it.

5. A mutant according to claim 4, wherein the animal is a transgenic animal expressing the mutant as a result of transformation with a transgene.

6. A mutant according to claim 4 or claim 5, which comprises the sequence PRPRSFSAPFSSQDS, or a sequence substantially homologous thereto.

7. A mutant according to any one of claims 4 to 6 which comprises the sequence MLRALNRLAARPGGQPPTLLLLPVRGPRPRSFSAPFSSQDS, or a sequence substantially homologous thereto.

8. A nucleic acid encoding a 5'OT-EST polypeptide or mutant 5'OT-EST polypeptide according to any preceding claim.

9. A nucleic acid according to claim 8, having a sequence selected from the group consisting of any one of SEQ. ID. Nos. 1, 3, 5, 7, 16 or 17; sequences which are

hybridisable under stringent conditions with an oligonucleotide comprising 20 contiguous bases from any one of SEQ. ID. Nos. 1, 3, 5, 7, 16 or 17; sequences substantially homologous to any one of SEQ. ID. Nos. 1, 3, 5, 7, 16 or 17; and sequences complementary thereto.

10. A nucleic acid according to claim 9, comprising the sequence ATGTTGCGGGCTT TGAACCGCCTGGCCGCGCGGCCCGGGGGCCAGCCCCCAACCCTGCTCCTTCTGCCCGTGC GCGGCCCACGGCCCCGCTCATTCTCGGCTCCTTTTTCCTCGCAGGATAGC, or an equivalent sequence which encodes the same polypeptide having regard to the degeneracy of the nucleic acid code, or a sequence substantially homologous thereto.

11. A nucleic acid vector comprising a nucleic acid sequence according to any one of claims 8 to 11.

12. A vector according to claim 11 which is a cosmid vector.

13. A vector according to claim 11 or claim 12 further comprising the sequences of the oxytocin (OT) gene, the vasopressin (AVP) gene and/or the human growth hormone (hGH) gene.

14. A vector according to claim 12 having the structure of cVO14 as set forth in Figure 4 (SEQ. ID. No. 17).

15. A cell transformed with a vector according to any one of claims 11 to 13.

16. A method for producing a 5'OT-EST polypeptide or a mutant 5'OT-EST polypeptide according to any one of claims 1 to 7, comprising transforming a cell with a vector according to any one of claims 11 to 13 and culturing the cell to produce the polypeptide.

17. A transgenic non-human animal expressing, as a result of transgene expression, a 5'OT-EST polypeptide or mutant 5'OT-EST polypeptide according to any one of claim 1 to 7.

18. A transgenic animal according to claim 17, which has been transformed with a vector according to any one of claims 12 to 14.

19. A transgenic animal according to claim 17 or claim 18, comprising more than one copy of the transgene.

20. A transgenic animal according to any one of claims 17 to 19, which is a mammal.

21. A transgenic animal according to claim 20 which is a rat.

22. A transgenic rat comprising at least four concatameric copies of a transgene having the structure of cVO14 as set forth in Figure 4 (SEQ. ID. No. 17).

23. A non-human mammal possessing the following obese phenotype: (i) a very late onset of obesity, (ii) a highly selective visceral distribution of fat developing on a normal rodent diet, without hyperphagia, (iii) an effect greatly preponderant in males, (iv) a predisposition to excessive dietary-fat induced obesity at an early age, before the phenotype becomes apparent on a normal diet, and (v) a dominant pattern of inheritance; the non-human mammal being obtainable by transformation with a vector according to any one of claims 11 to 14.

24. Use of an animal according to any one of claims 17 to 23 as a model for human late onset obesity, human dietary-fat associated juvenile obesity, human female post-menopausal obesity and/or human male infertility.

25. A method for identifying a compound or compounds capable of modulating obesity and/or infertility in a mammal, comprising the steps of:

a) exposing an animal according to any one of claims 17 to 24 to the compound or compounds to be tested;

b) determining the effect of the compound on the obesity and/or infertility phenotype; and

c) selecting the compound or compounds which are capable of modulating the obesity and/or infertility phenotype in the desired manner.

26. A method for producing a compound or compounds capable of modulating obesity and/or infertility in a mammal, comprising the steps of:

a) exposing an animal according to any one of claims 17 to 24 to the compound or compounds to be tested;

b) determining the effect of the compound on the obesity and/or infertility phenotype;

c) selecting the compound or compounds which are capable of modulating the obesity and/or infertility phenotype in the desired manner; and

d) producing the compound or compounds by conventional isolation or synthesis techniques.

27. A method for identifying a candidate compound capable of influencing lipid transport, comprising the steps of:

a) contacting 5'OT-EST polypeptide with a candidate compound or compounds and determining which candidate compound or compounds is capable of interacting with 5'OT-EST;

b) optionally, testing candidate compounds which interact with 5'OT-EST in a method according to claim 25.

28. A diagnostic reagent for the detection of mutations, polymorphisms or other changes in *5'OT-EST* which may predispose an individual to obesity.

29. Use of a tissue derived from a transgenic animal according to any one of claims 17 to 24 in a screen to identify a genetic cause of obesity, comprising the steps of:

a) isolating one or more gene products from tissue derived from a transgenic animal according to any one of claims 17 to 24; and

b) determining whether the expression of a gene product is correlated with obesity.

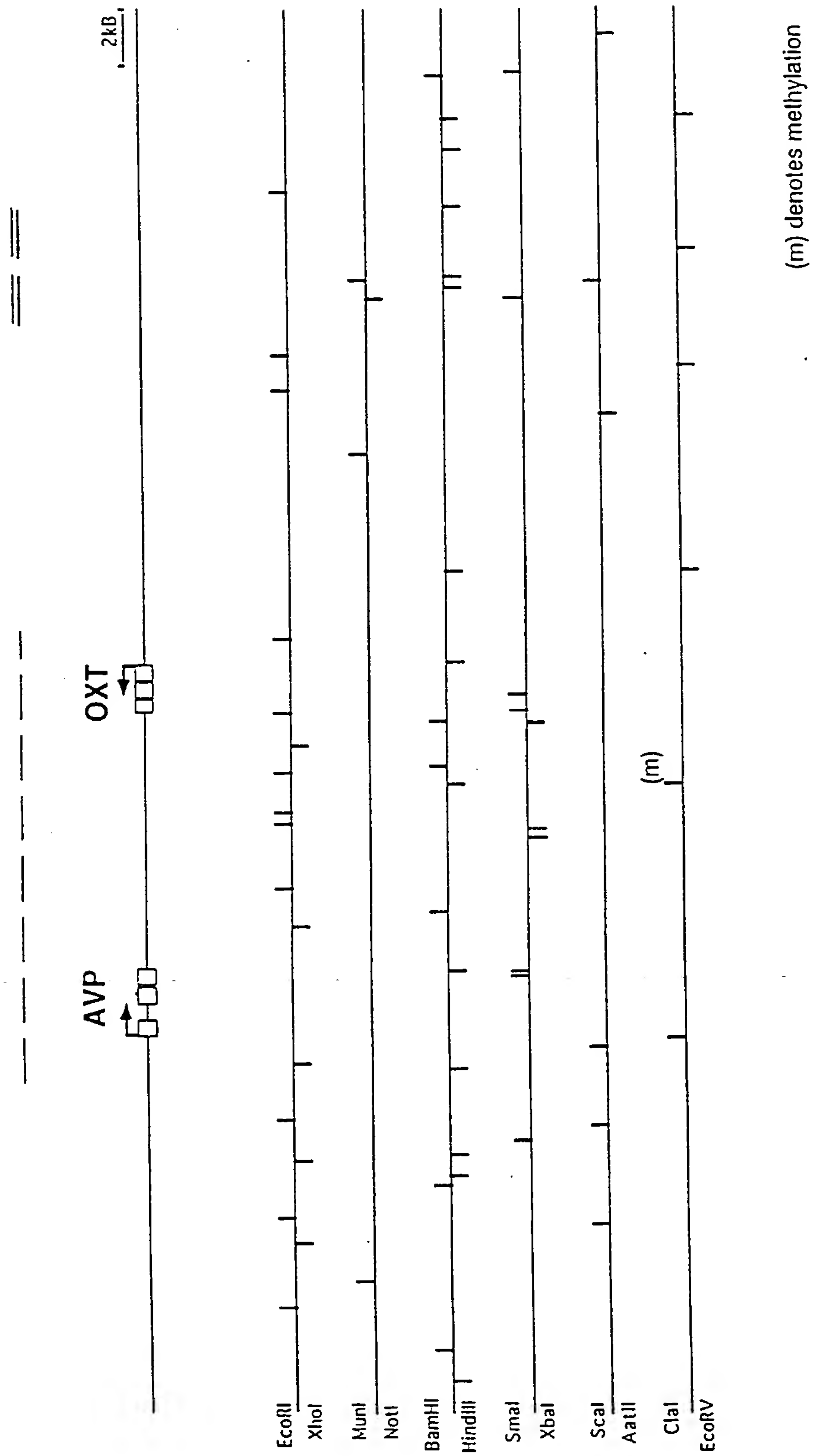
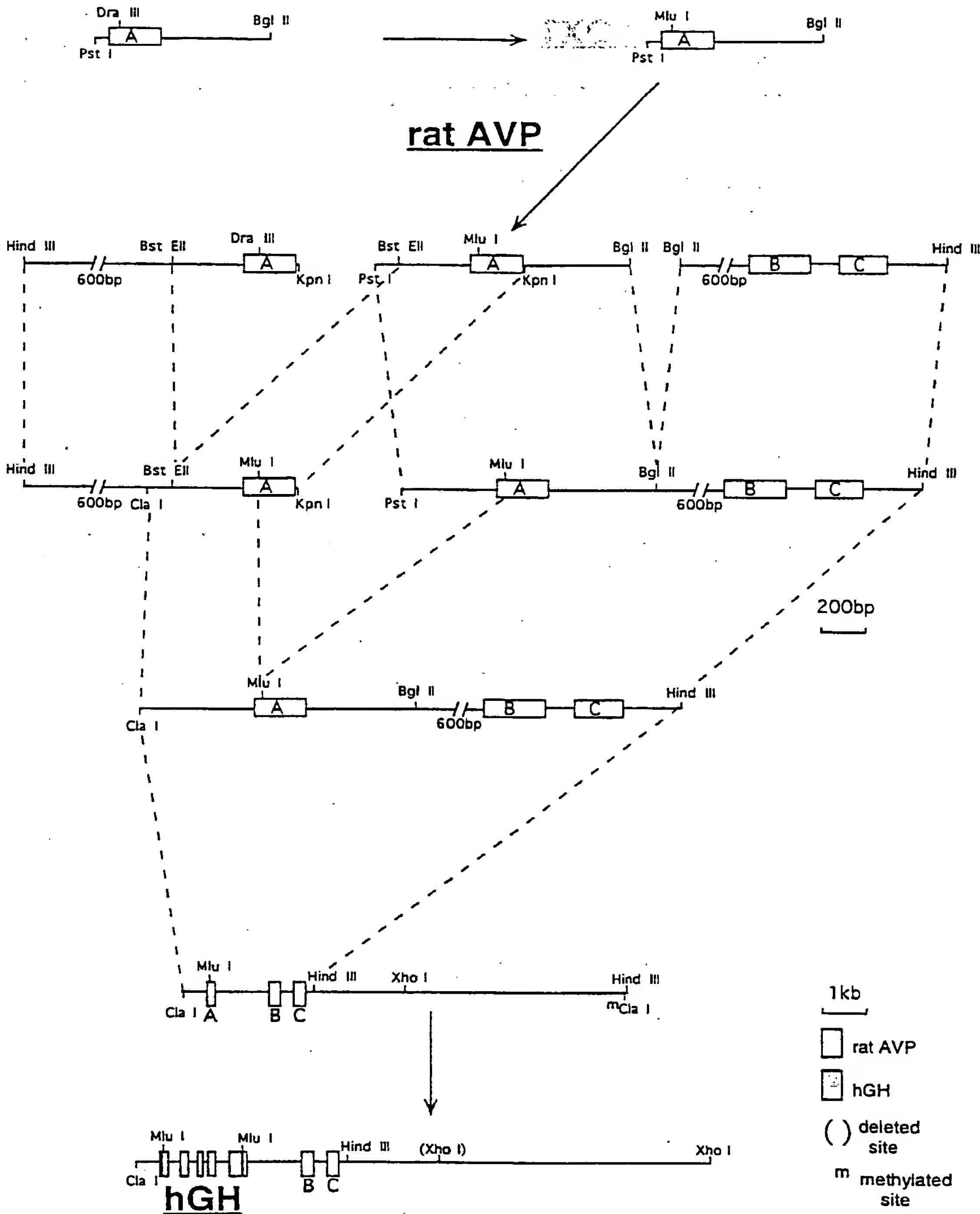

2kB
AVP
OXT
EcoRI
XhoI
MunI
NotI
BamHI
HindIII
SmaI
XbaI
ScaI
AatII
ClaI
EcoRV
(m)
(m) denotes methylation

FIG. 1



Dra III
Bgl II
Pst I
A
Mlu I
rat AVP
Hind III
Bst EII
600bp
Kpn I
Pst I
B
C
Cla I
200bp
Xho I
mCla I
1kb
rat AVP
hGH
( ) deleted site
m methylated site
(Xho I)

FIG. 2

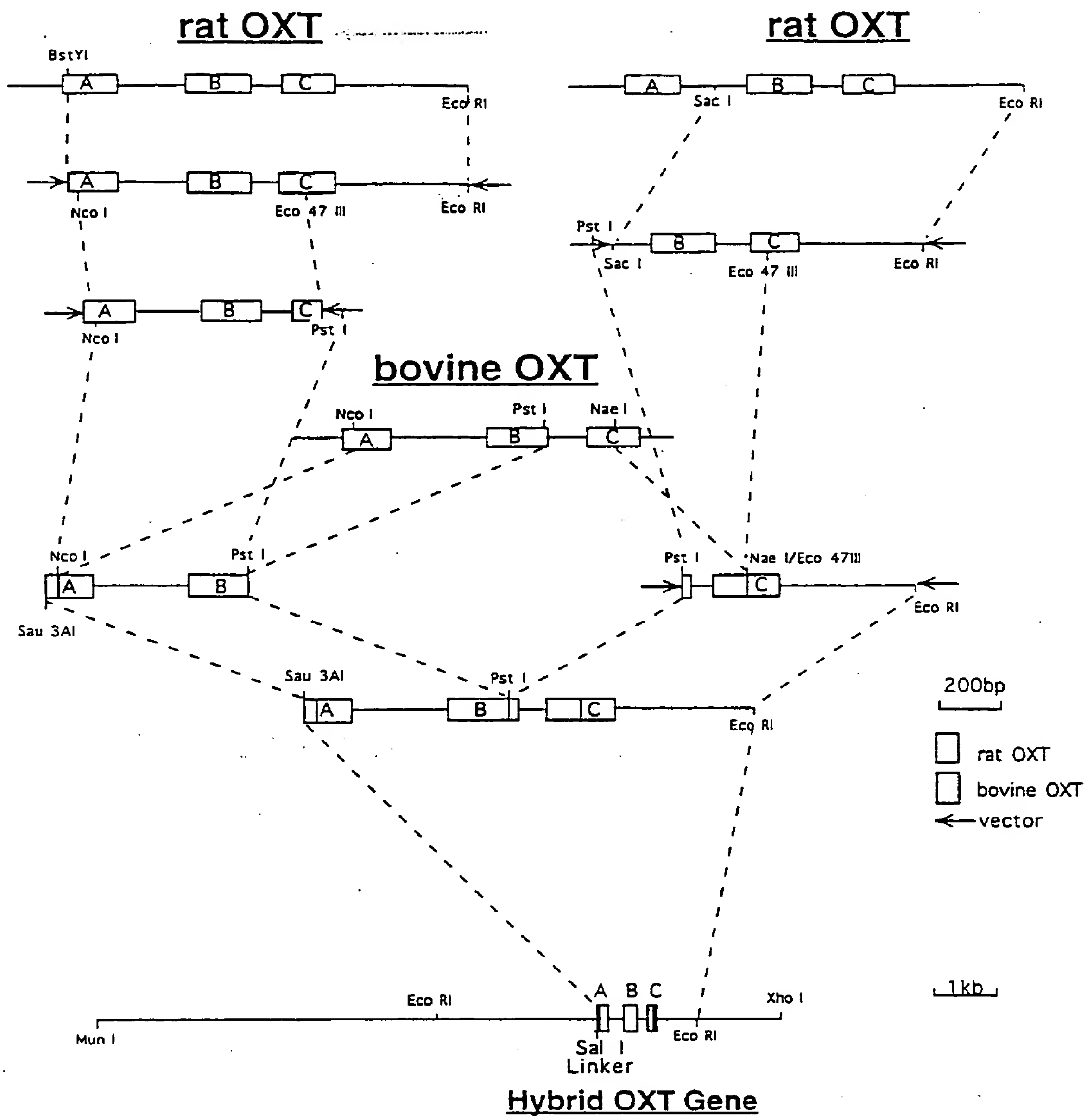

rat OXT
rat OXT
BstYI
A
B
C
Eco RI
Nco I
Eco 47 III
Sac I
Pst I
Pst I
bovine OXT
Nae I
Sau 3AI
Nae I/Eco 47III
200bp
rat OXT
bovine OXT
vector
1kb
Mun I
Xho I
Sal I
Linker
Hybrid OXT Gene

FIG. 3

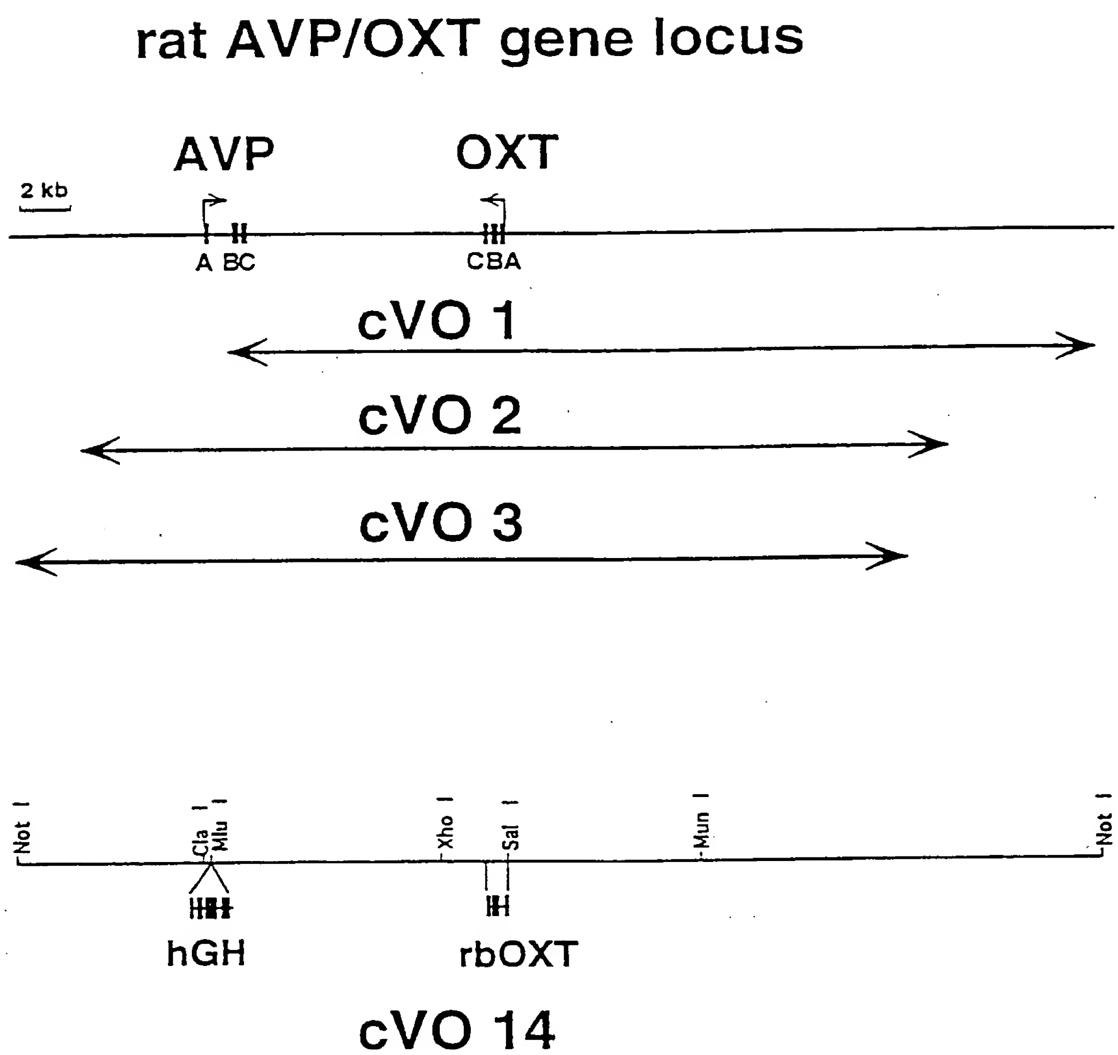

rat AVP/OXT gene locus
AVP
OXT
2 kb
A BC
CBA
cVO 1
cVO 2
cVO 3
Not
Cla
Mlu
Xho
Sal
Mun
Not
hGH
rbOXT
cVO 14

FIG. 4

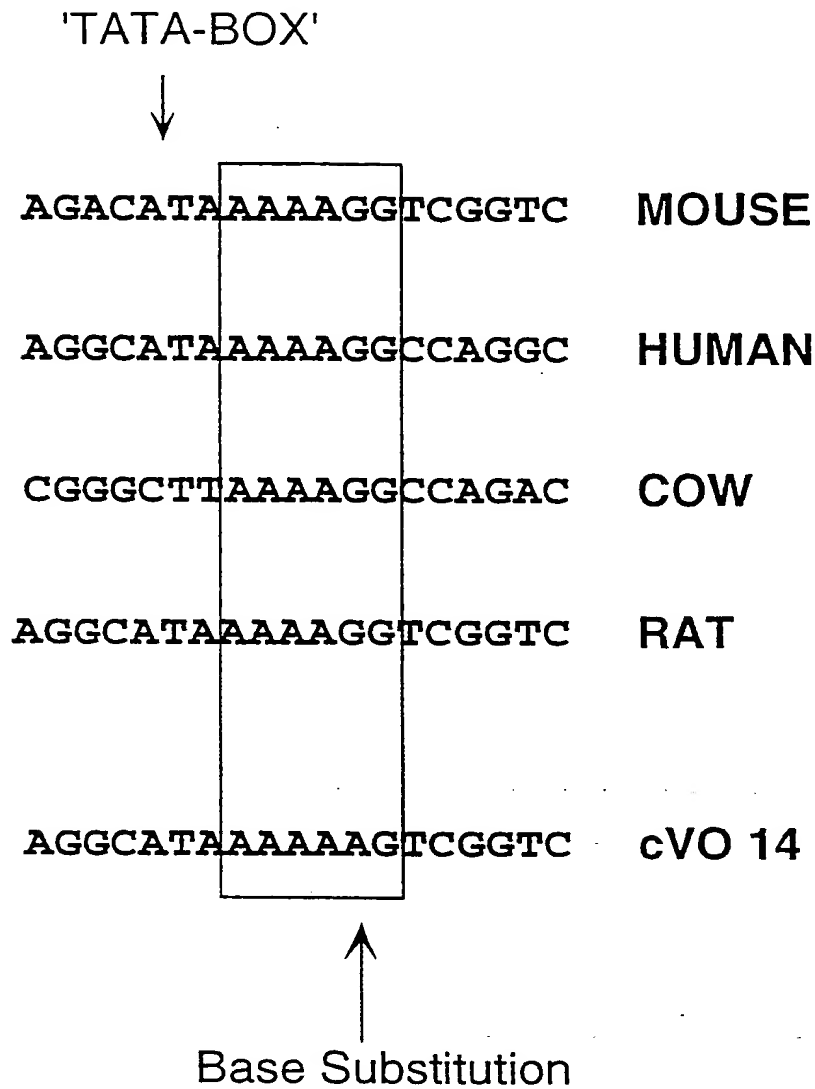

'TATA-BOX'
AGACATAAAAAGGTCGGTC MOUSE
AGGCATAAAAAGGCCAGGC HUMAN
CGGGCTTAAAAGGCCAGAC COW
AGGCATAAAAAGGTCGGTC RAT
AGGCATAAAAAAGTCGGTC cVO 14
Base Substitution

FIG. 5

Figure 6 5'OT-EST PROTEIN OF DIFFERENT SPECIES

Mouse
MLRALNRLAQRPGDRPPTPLLLPVRGRKTRHDPPAKSKVGRVQTPPAVDPAEFFVLTERY
GQYRETVRALRLEFTLDVRRKLHEARAGVLAERKAQQAITEHRELMAWNRDENRRMQELR
IARLQLEAQAQEVQKAEAQRQRAQEEQAWVQLKEQEVLKLQEEAKNFITRENLEARIEEA
LDSPKSYNWAVTKEGQVVRN

Rat
MLRALNRLAARPGGQPPTLLLLPVRGRKTRHDPPAKSKVGRVKMPPAVDPAELFVLTERY
RQYRETVRALRREFTLEVRGKLHEARAGVLAERKAQEAIREHQELMAWNREENRRLQELR
IARLQLEAQAQELRQAEVQAQRAQEEQAWVQLKEQEVLKLQEEAKNFITRENLEARIEEA
LDSPKSYNWAVTKEGQVVRN

Human
MLRALSRLGAGTPCRPRAPLVLPARGRKTRHDPLAKSKIERVNMPPAVDPAEFFVLMERY
QHYRQTVRALRMEFVSEVQRKVHEARAGVLAERKALKDAAEHRELMAWNQAENRRLHELR
IARLRQEEREQEQRQALEQARKAEEVQAWAQRKEREVLQLQEEVKNFITRENLEARVEAA
LDSRKNYNWAITREGLVVRPQRRDS

<u>Alignment</u>

```
Mouse   MLRALNRLAQRPGDRPPTPLLLPVRGRKTRHDPPAKSKVGRVQTPPAVDPAEFFVLTERY
Rat     MLRALNRLAARPGGQPPTLLLLPVRGRKTRHDPPAKSKVGRVKMPPAVDPAELFVLTERY
Human   MLRALSRLGAGTPCRPRAPLVLPARGRKTRHDPLAKSKIERVNMPPAVDPAEFFVLMERY

Mouse   GQYRETVRALRLEFTLDVRRKLHEARAGVLAERKAQQAITEHRELMAWNRDENRRMQELR
Rat     RQYRETVRALRREFTLEVRGKLHEARAGVLAERKAQEAIREHQELMAWNREENRRLQELR
Human   QHYRQTVRALRMEFVSEVQRKVHEARAGVLAERKALKDAAEHRELMAWNQAENRRLHELR

Mouse   IARLQLEAQAQEVQKAEAQRQRAQEEQAWVQLKEQEVLKLQEEAKNFITRENLEARIEEA
Rat     IARLQLEAQAQELRQAEVQAQRAQEEQAWVQLKEQEVLKLQEEAKNFITRENLEARIEEA
Human   IARLRQEEREQEQRQALEQARKAEEVQAWAQRKEREVLQLQEEVKNFITRENLEARVEAA

Mouse   LDSPKSYNWAVTKEGQVVRN
Rat     LDSPKSYNWAVTKEGQVVRN
Human   LDSRKNYNWAITREGLVVRPQRRDS
```

<u>Predicted deleted form in JP17</u>

MLRALNRLAARPGGQPPTLLLLPVRGprprsfsapfssqds

↑

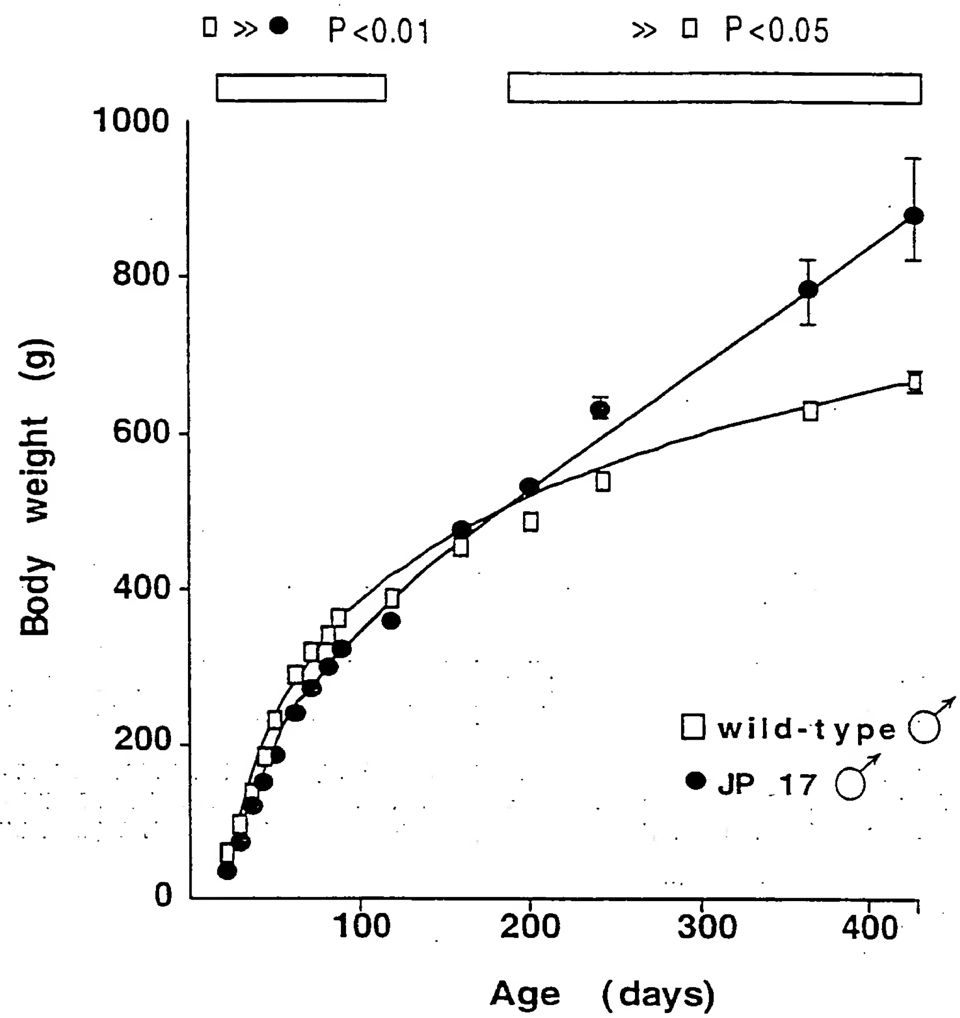

□ ≫ ● P<0.01
≫ □ P<0.05
1000
800
600
400
200
0
Body weight (g)
100
200
300
400
Age (days)
□ wild-type ♂
● JP_17 ♂

FIG. 7

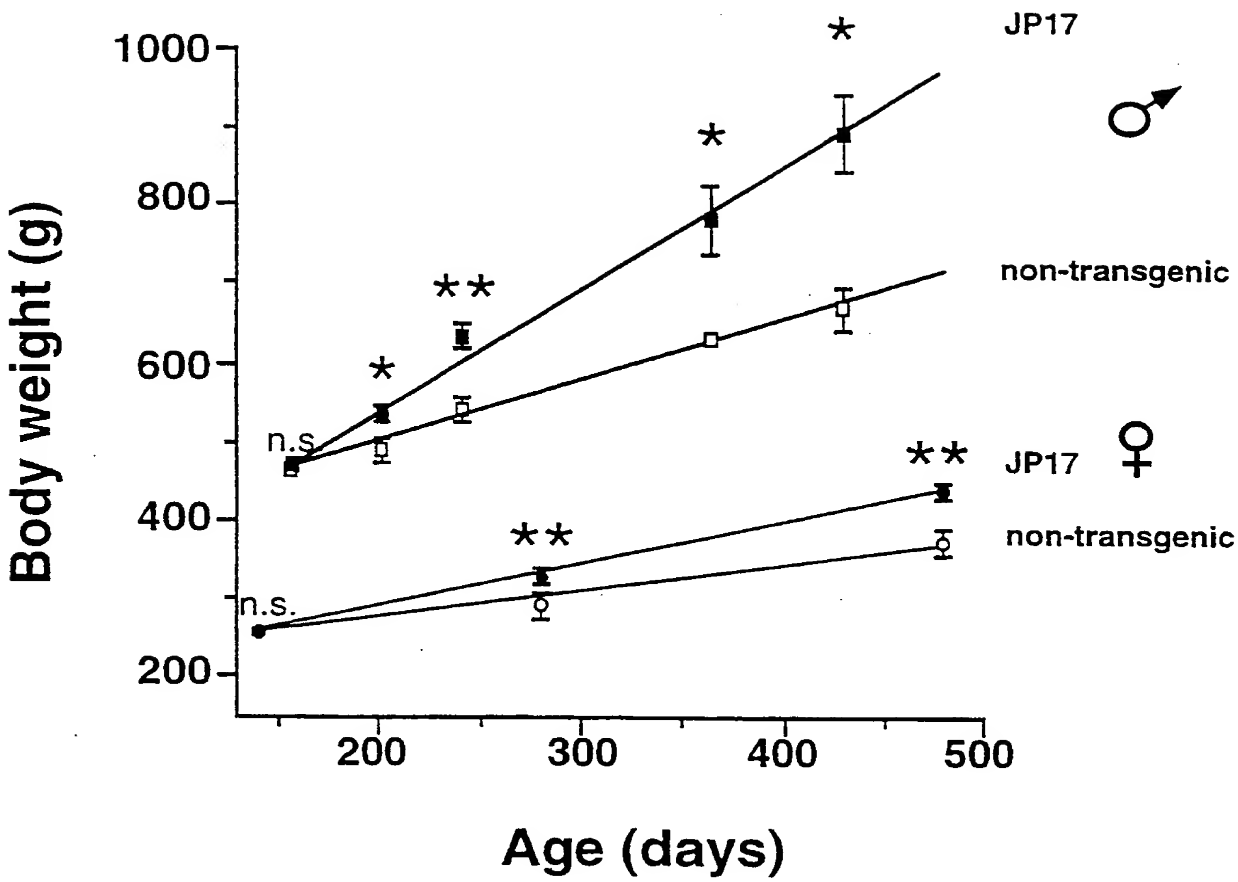
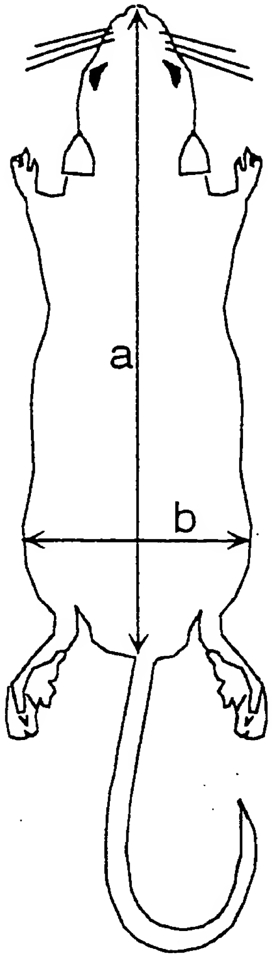

JP17
non-transgenic
JP17
non-transgenic
Body weight (g)
1000
800
600
400
200
200
300
400
500
Age (days)
n.s.
n.s.
*
**
*
*
**
**

FIG. 8



20 weeks

	non-transgenic	JP17 transgenic
a	260.33 ± 0.28	243.34 ± 0.13 ***
b	92.67 ± 0.29	115.14 ± 0.24***

52 weeks

	non-transgenic	JP17 transgenic
a	273.83 ± 0.28	261.0 ± 0.45 ns
b	113.83 ± 0.10	157.83 ± 0.61 ***

FIG. 9

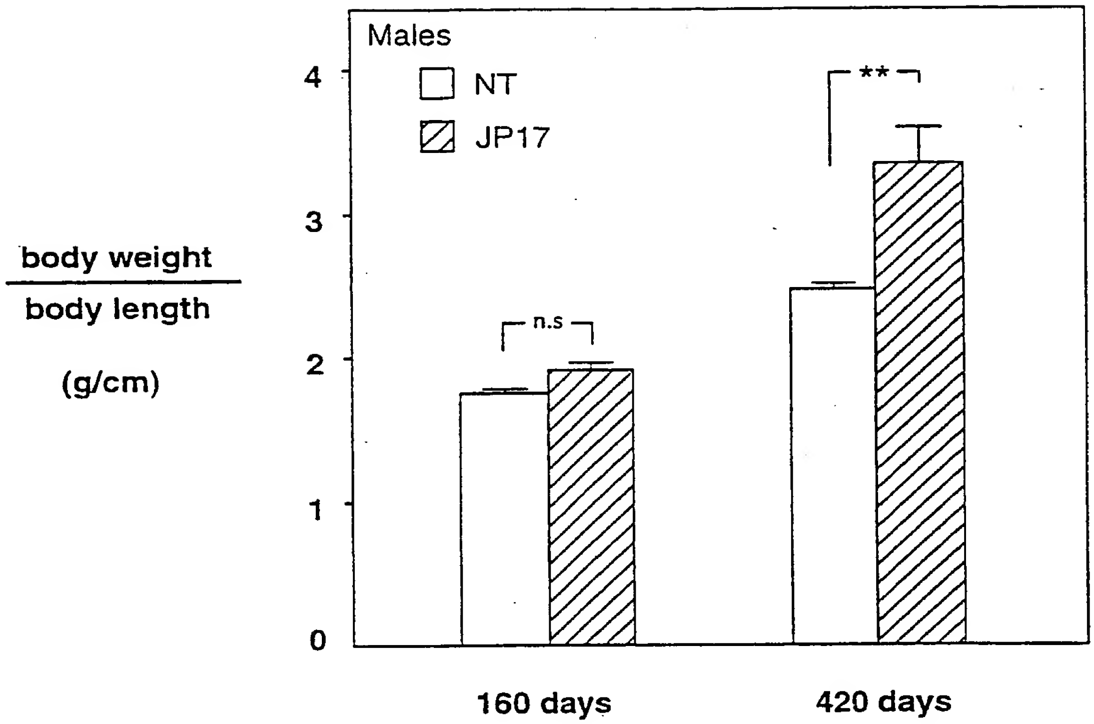

Males
NT
JP17
**
n.s
4
3
2
1
0
body weight
body length
(g/cm)
160 days
420 days

FIG. 10

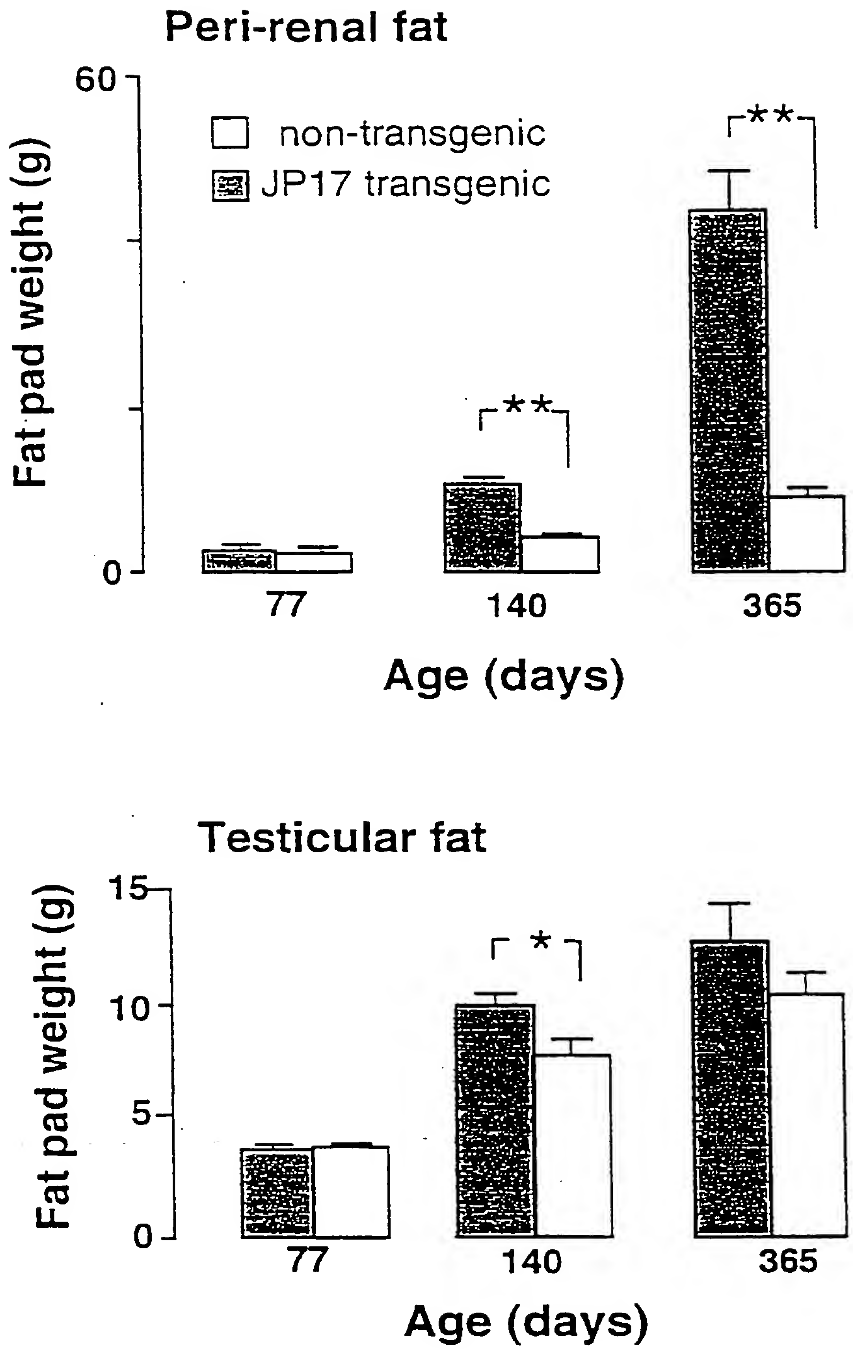

Peri-renal fat
60
0
non-transgenic
JP17 transgenic
**
**
Fat pad weight (g)
77
140
365
Age (days)
Testicular fat
15
10
5
0
*
Fat pad weight (g)
77
140
365
Age (days)

FIG. 11

11/14

	Cholesterol mg/dl	Triglyceride mg/dl	Glucose mg/dl	Insulin ng/ml	Leptin ng/ml	Corticosterone ng/ml
MALE TRANSGENIC	122.3 +/- 6.4	*295.6 +/- 28.7	114.7 +/- 4.2	1.94 +/- 0.89	*24.4 +/- 1.49	168.9 +/- 23.5
MALE NON-TRANSGENIC	129.9 +/- 9.3	178.9 +/- 23.5	121.0 +/- 3.9	2.8 +/- 1.93	9.51 +/- 2.14	113.9 +/- 20.3
FEMALE TRANSGENIC	94.9 +/- 5.9	224.2 +/- 52.3	126.3 +/- 3.3	2.51 +/- 0.64	*14.74 +/- 1.38	256.3 +/- 104.1
FEMALE NON-TRANSGENIC	100.2 +/- 8.0	195.5 +/- 34.5	135.4 +/- 6.7	2.54 +/- 2.32	4.58 +/- 0.47	349.3 +/- 123.7

FIG. 12

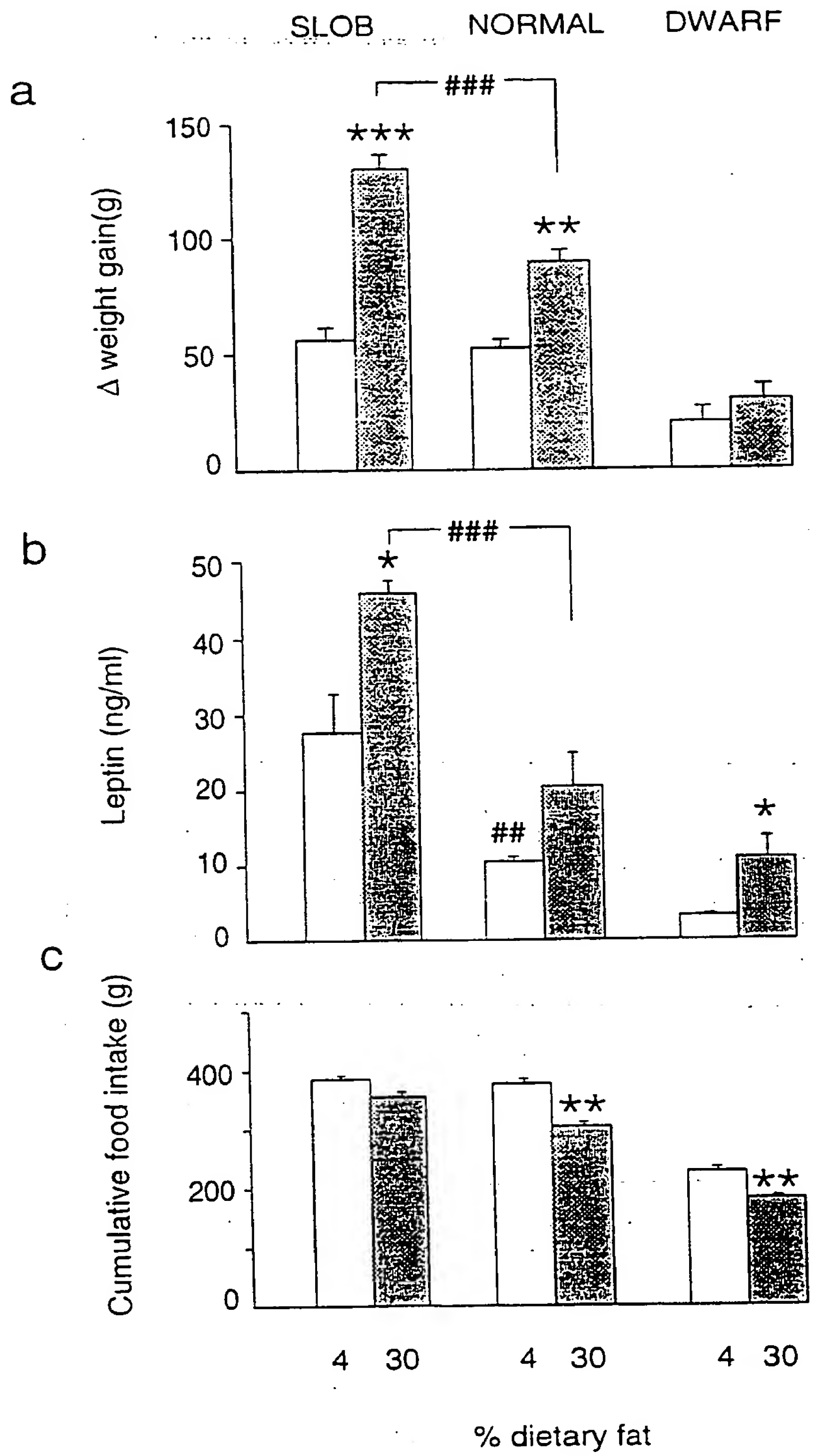

SLOB
NORMAL
DWARF
a
###
150
***
100
**
50
0
Δ weight gain(g)
b
###
50
*
40
30
20
##
*
10
0
Leptin (ng/ml)
c
400
**
200
**
0
Cumulative food intake (g)
4
30
4
30
4
30
% dietary fat

FIG. 13

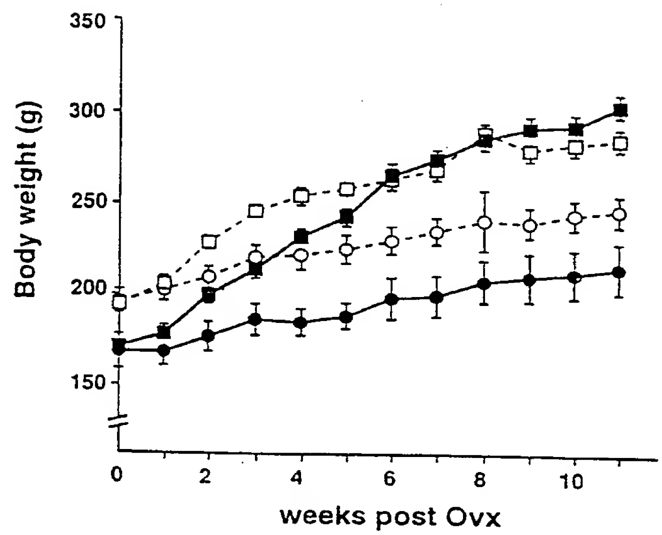


FIG 14

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MEDICAL RESEARCH COUNCIL
(B) STREET: 20 PARK CRESCENT
(C) CITY: LONDON
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1N 4AL

(ii) TITLE OF INVENTION: GENE

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 924 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:5..604

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
TGTC ATG TTG CGG GCT TTG AAC CGC CTG GCC GCG CGG CCC GGG GGC CAG      49
     Met Leu Arg Ala Leu Asn Arg Leu Ala Ala Arg Pro Gly Gly Gln
       1               5                  10                  15

CCC CCA ACC CTG CTC CTT CTG CCC GTG CGC GGC CGC AAG ACC CGC CAC       97
Pro Pro Thr Leu Leu Leu Leu Pro Val Arg Gly Arg Lys Thr Arg His
                 20                  25                  30

GAT CCG CCT GCC AAG TCC AAG GTC GGG CGC GTG AAA ATG CCT CCT GCA      145
Asp Pro Pro Ala Lys Ser Lys Val Gly Arg Val Lys Met Pro Pro Ala
             35                  40                  45

GTG GAC CCT GCG GAA TTG TTC GTG TTG ACC GAG CGC TAC CGA CAG TAC      193
Val Asp Pro Ala Glu Leu Phe Val Leu Thr Glu Arg Tyr Arg Gln Tyr
         50                  55                  60
```

CGG GAG ACG GTG CGC GCT CTC AGG CGA GAG TTC ACA TTG GAG GTG CGA 241
Arg Glu Thr Val Arg Ala Leu Arg Arg Glu Phe Thr Leu Glu Val Arg
65 70 75

GGG AAA TTG CAC GAG GCC CGA GCC GGG GTT CTG GCT GAG CGC AAG GCG 289
Gly Lys Leu His Glu Ala Arg Ala Gly Val Leu Ala Glu Arg Lys Ala
80 85 90 95

CAA GAG GCC ATC AGA GAG CAC CAG GAG CTG ATG GCC TGG AAC CGG GAG 337
Gln Glu Ala Ile Arg Glu His Gln Glu Leu Met Ala Trp Asn Arg Glu
100 105 110

GAG AAC CGG AGA CTG CAG GAA CTA CGG ATA GCT AGG TTG CAG CTC GAA 385
Glu Asn Arg Arg Leu Gln Glu Leu Arg Ile Ala Arg Leu Gln Leu Glu
115 120 125

GCA CAG GCC CAG GAG CTG CGG CAG GCT GAG GTC CAG GCC CAG AGG GCC 433
Ala Gln Ala Gln Glu Leu Arg Gln Ala Glu Val Gln Ala Gln Arg Ala
130 135 140

CAG GAG GAG CAG GCT TGG GTG CAA CTG AAA GAA CAA GAA GTT CTC AAA 481
Gln Glu Glu Gln Ala Trp Val Gln Leu Lys Glu Gln Glu Val Leu Lys
145 150 155

CTG CAG GAG GAG GCC AAA AAC TTC ATC ACT CGG GAG AAC CTG GAG GCA 529
Leu Gln Glu Glu Ala Lys Asn Phe Ile Thr Arg Glu Asn Leu Glu Ala
160 165 170 175

CGG ATA GAA GAG GCC TTG GAC TCT CCG AAG AGT TAT AAC TGG GCG GTC 577
Arg Ile Glu Glu Ala Leu Asp Ser Pro Lys Ser Tyr Asn Trp Ala Val
180 185 190

ACC AAA GAA GGG CAG GTG GTC AGG AAC TGAGAACAGA GGCCTCTCAG 624
Thr Lys Glu Gly Gln Val Val Arg Asn
195 200

GCCCAAATAA GGACAGTGCT TGCCTAGGGA CTGGATATTG GGGTAGAAAT TGGTGCATCC 684

CAGGAGGGTG GCACAGCCTT GTCCAGAGCA GCCCCCATTC ATTCTAGATT TGGCACCAGG 744

TATAGTACCT GTTCTGACAC CACATACAAA CTCCGGACAG CATTAAACTC TGGGAAGTTC 804

CTATCACACA GAAGATCAGA CTGGACTGTC CCCTCTAGAA GCCAAGAGCT GTCTCCTGAG 864

TTTCTTGGAA TAGTGTGAGC CCAATGTTTC CTGCTTTTAT AAATAAACTA TTGGAAAGCA 924

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Leu Arg Ala Leu Asn Arg Leu Ala Ala Arg Pro Gly Gly Gln Pro
1 5 10 15

```
Pro Thr Leu Leu Leu Leu Pro Val Arg Gly Arg Lys Thr Arg His Asp
            20                  25                  30

Pro Pro Ala Lys Ser Lys Val Gly Arg Val Lys Met Pro Pro Ala Val
        35                  40                  45

Asp Pro Ala Glu Leu Phe Val Leu Thr Glu Arg Tyr Arg Gln Tyr Arg
    50                  55                  60

Glu Thr Val Arg Ala Leu Arg Arg Glu Phe Thr Leu Glu Val Arg Gly
65                  70                  75                  80

Lys Leu His Glu Ala Arg Ala Gly Val Leu Ala Glu Arg Lys Ala Gln
                85                  90                  95

Glu Ala Ile Arg Glu His Gln Glu Leu Met Ala Trp Asn Arg Glu Glu
            100                 105                 110

Asn Arg Arg Leu Gln Glu Leu Arg Ile Ala Arg Leu Gln Leu Glu Ala
        115                 120                 125

Gln Ala Gln Glu Leu Arg Gln Ala Glu Val Gln Ala Gln Arg Ala Gln
    130                 135                 140

Glu Glu Gln Ala Trp Val Gln Leu Lys Glu Gln Glu Val Leu Lys Leu
145                 150                 155                 160

Gln Glu Glu Ala Lys Asn Phe Ile Thr Arg Glu Asn Leu Glu Ala Arg
                165                 170                 175

Ile Glu Glu Ala Leu Asp Ser Pro Lys Ser Tyr Asn Trp Ala Val Thr
            180                 185                 190

Lys Glu Gly Gln Val Val Arg Asn
        195                 200
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 998 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..615

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Met Leu Arg Ala Leu Ser Arg Leu Gly Ala Gly Thr Pro Cys Arg Pro
```

205 210 215

CGG GCC CCT CTG GTG CTG CCA GCG CGC GGC CGC AAG ACC CGC CAC GAC 96
Arg Ala Pro Leu Val Leu Pro Ala Arg Gly Arg Lys Thr Arg His Asp
220 225 230

CCG CTG GCC AAA TCC AAG ATC GAG CGA GTG AAC ATG CCG CCC GCG GTG 144
Pro Leu Ala Lys Ser Lys Ile Glu Arg Val Asn Met Pro Pro Ala Val
235 240 245

GAC CCT GCG GAG TTC TTC GTG CTG ATG GAG CGT TAC CAG CAC TAC CGC 192
Asp Pro Ala Glu Phe Phe Val Leu Met Glu Arg Tyr Gln His Tyr Arg
250 255 260

CAG ACC GTG CGC GCC CTC AGG ATG GAG TTC GTG TCC GAG GTG CAG AGG 240
Gln Thr Val Arg Ala Leu Arg Met Glu Phe Val Ser Glu Val Gln Arg
265 270 275 280

AAG GTG CAC GAG GCC CGA GCC GGG GTT CTG GCG GAG CGC AAG GCC CTG 288
Lys Val His Glu Ala Arg Ala Gly Val Leu Ala Glu Arg Lys Ala Leu
285 290 295

AAG GAC GCC GCC GAG CAC CGC GAG CTG ATG GCC TGG AAC CAG GCG GAG 336
Lys Asp Ala Ala Glu His Arg Glu Leu Met Ala Trp Asn Gln Ala Glu
300 305 310

AAC CGG CGG CTG CAC GAG CTG CGG ATA GCG AGG CTG CGG CAG GAG GAG 384
Asn Arg Arg Leu His Glu Leu Arg Ile Ala Arg Leu Arg Gln Glu Glu
315 320 325

CGG GAG CAG GAG CAG CGG CAG GCG TTG GAG CAG GCC CGC AAG GCC GAA 432
Arg Glu Gln Glu Gln Arg Gln Ala Leu Glu Gln Ala Arg Lys Ala Glu
330 335 340

GAG GTG CAG GCC TGG GCG CAG CGC AAG GAG CGG GAA GTG CTG CAG CTG 480
Glu Val Gln Ala Trp Ala Gln Arg Lys Glu Arg Glu Val Leu Gln Leu
345 350 355 360

CAG GAA GAG GTG AAA AAC TTC ATC ACC CGA GAG AAC CTG GAG GCA CGG 528
Gln Glu Glu Val Lys Asn Phe Ile Thr Arg Glu Asn Leu Glu Ala Arg
365 370 375

GTG GAA GCA GCA TTG GAC TCC CGG AAG AAC TAC AAC TGG GCC ATC ACC 576
Val Glu Ala Ala Leu Asp Ser Arg Lys Asn Tyr Asn Trp Ala Ile Thr
380 385 390

AGA GAG GGG CTG GTG GTC AGG CCA CAA CGC AGG GAC TCC TAGGGGCCCA 625
Arg Glu Gly Leu Val Val Arg Pro Gln Arg Arg Asp Ser
395 400 405

GTAAGGACAG TGCCCGCCAG GGACCATGTA TGTATCATGG CGGAAGAGTT GGCCCTGACC 685

TGGAATAAAG CAGTTGGTGT TGCTTATGAG GAAGGTTCAG CCTTATCCAG CACAGCCTTC 745

ACGTTTTGCC CTCTGCTGTC ACCACTTGGT CAGAAACTTC CAAACGCAGT GCCCTGTTCT 805

GCCGGTGTGT AAAGCCTCAG CGCACCAGGA GACCCTAGAG TGGTTTCCAT CTCACAGAGA 865

ATCAGACAGG CCACAGCCCC CTCAGGCAGC CAGGTCATCT GAGTATCATT AAGAGTAGTG 925

ATGGGAAGAT TACAGTCTGA GGGCCAAACG TGCCTGCTTC CTGTTTTTGT AAATAAAGTT 985

TTGTTGGAAC ACA 998

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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Met Leu Arg Ala Leu Ser Arg Leu Gly Ala Gly Thr Pro Cys Arg Pro
  1               5                  10                  15

Arg Ala Pro Leu Val Leu Pro Ala Arg Gly Arg Lys Thr Arg His Asp
             20                  25                  30

Pro Leu Ala Lys Ser Lys Ile Glu Arg Val Asn Met Pro Pro Ala Val
         35                  40                  45

Asp Pro Ala Glu Phe Phe Val Leu Met Glu Arg Tyr Gln His Tyr Arg
     50                  55                  60

Gln Thr Val Arg Ala Leu Arg Met Glu Phe Val Ser Glu Val Gln Arg
 65                  70                  75                  80

Lys Val His Glu Ala Arg Ala Gly Val Leu Ala Glu Arg Lys Ala Leu
                 85                  90                  95

Lys Asp Ala Ala Glu His Arg Glu Leu Met Ala Trp Asn Gln Ala Glu
            100                 105                 110

Asn Arg Arg Leu His Glu Leu Arg Ile Ala Arg Leu Arg Gln Glu Glu
        115                 120                 125

Arg Glu Gln Glu Gln Arg Gln Ala Leu Glu Gln Ala Arg Lys Ala Glu
    130                 135                 140

Glu Val Gln Ala Trp Ala Gln Arg Lys Glu Arg Glu Val Leu Gln Leu
145                 150                 155                 160

Gln Glu Glu Val Lys Asn Phe Ile Thr Arg Glu Asn Leu Glu Ala Arg
                165                 170                 175

Val Glu Ala Ala Leu Asp Ser Arg Lys Asn Tyr Asn Trp Ala Ile Thr
            180                 185                 190

Arg Glu Gly Leu Val Val Arg Pro Gln Arg Arg Asp Ser
        195                 200                 205
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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 943 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:5..604

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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     Met Leu Arg Ala Leu Asn Arg Leu Ala Gln Arg Pro Gly Asp Arg
                     210                 215                 220

CCC CCG ACC CCG CTG CTC CTG CCC GTG CGC GGC CGC AAG ACC CGC CAT       97
Pro Pro Thr Pro Leu Leu Leu Pro Val Arg Gly Arg Lys Thr Arg His
                225                 230                 235

GAC CCG CCT GCC AAA TCC AAG GTC GGA CGG GTG CAG ACG CCT CCC GCC      145
Asp Pro Pro Ala Lys Ser Lys Val Gly Arg Val Gln Thr Pro Pro Ala
            240                 245                 250

GTG GAC CCT GCG GAA TTC TTC GTG TTG ACC GAG CGC TAC GGA CAG TAC      193
Val Asp Pro Ala Glu Phe Phe Val Leu Thr Glu Arg Tyr Gly Gln Tyr
        255                 260                 265

CGG GAG ACC GTG CGC GCT CTC AGG CTA GAG TTC ACG TTG GAT GTG CGA      241
Arg Glu Thr Val Arg Ala Leu Arg Leu Glu Phe Thr Leu Asp Val Arg
    270                 275                 280

AGG AAA TTG CAC GAG GCC CGA GCC GGG GTT CTG GCC GAG CGC AAG GCG      289
Arg Lys Leu His Glu Ala Arg Ala Gly Val Leu Ala Glu Arg Lys Ala
285                 290                 295                 300

CAG CAG GCC ATC ACG GAG CAC CGG GAG CTG ATG GCC TGG AAC CGG GAC      337
Gln Gln Ala Ile Thr Glu His Arg Glu Leu Met Ala Trp Asn Arg Asp
                305                 310                 315

GAG AAC CGG CGA ATG CAG GAG CTA CGG ATA GCG AGG TTG CAG CTG GAA      385
Glu Asn Arg Arg Met Gln Glu Leu Arg Ile Ala Arg Leu Gln Leu Glu
            320                 325                 330

GCA CAG GCC CAG GAG GTG CAG AAG GCT GAG GCC CAG CGC CAG AGG GCT      433
Ala Gln Ala Gln Glu Val Gln Lys Ala Glu Ala Gln Arg Gln Arg Ala
        335                 340                 345

CAG GAG GAG CAG GCT TGG GTG CAA CTG AAA GAG CAA GAA GTG CTC AAG      481
Gln Glu Glu Gln Ala Trp Val Gln Leu Lys Glu Gln Glu Val Leu Lys
    350                 355                 360

CTG CAG GAG GAG GCA AAA AAC TTC ATC ACT CGG GAG AAC CTG GAG GCA      529
Leu Gln Glu Glu Ala Lys Asn Phe Ile Thr Arg Glu Asn Leu Glu Ala
365                 370                 375                 380

CGG ATA GAA GAA GCG TTG GAC TCT CCG AAG AGT TAC AAC TGG GCC GTC      577
Arg Ile Glu Glu Ala Leu Asp Ser Pro Lys Ser Tyr Asn Trp Ala Val
                385                 390                 395
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ACC AAA GAA GGG CAG GTG GTC AGG AAC TGAGCACAGA GACTTCTGGG 624
Thr Lys Glu Gly Gln Val Val Arg Asn
400 405

GGCCCAAATA AGCACAGTGC TTGCCTAGGG TCTGTGTACT GGGATAGGAA TTGGTACATC 684

CCAGGAGGAT GGCTCAGCCG TTTCCAGAGC AACCTCAGTC ACTCCAGGCT CGGCACTCAC 744

CACCTGACTG GGAACTCCCA GATGTCCCTG TTCTGGCACC ACAGTCAAAC TGAGGGCAGC 804

ATTAAACTCT GGGAAGTTCC TATCGCACAG AGGATCGGAC TGGACTGTGT CCCTCTAGAA 864

GCCAAGCTTG TCTTGTAAGT CTCTTGGAGT CCTGTGAGCC AAATGTTTCC TGCTTTTATA 924

AATAAAGTAT TGGAGCCCA 943

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Leu Arg Ala Leu Asn Arg Leu Ala Gln Arg Pro Gly Asp Arg Pro
1 5 10 15

Pro Thr Pro Leu Leu Leu Pro Val Arg Gly Arg Lys Thr Arg His Asp
20 25 30

Pro Pro Ala Lys Ser Lys Val Gly Arg Val Gln Thr Pro Pro Ala Val
35 40 45

Asp Pro Ala Glu Phe Phe Val Leu Thr Glu Arg Tyr Gly Gln Tyr Arg
50 55 60

Glu Thr Val Arg Ala Leu Arg Leu Glu Phe Thr Leu Asp Val Arg Arg
65 70 75 80

Lys Leu His Glu Ala Arg Ala Gly Val Leu Ala Glu Arg Lys Ala Gln
85 90 95

Gln Ala Ile Thr Glu His Arg Glu Leu Met Ala Trp Asn Arg Asp Glu
100 105 110

Asn Arg Arg Met Gln Glu Leu Arg Ile Ala Arg Leu Gln Leu Glu Ala
115 120 125

Gln Ala Gln Glu Val Gln Lys Ala Glu Ala Gln Arg Gln Arg Ala Gln
130 135 140

Glu Glu Gln Ala Trp Val Gln Leu Lys Glu Gln Glu Val Leu Lys Leu
145 150 155 160

Gln Glu Glu Ala Lys Asn Phe Ile Thr Arg Glu Asn Leu Glu Ala Arg
165 170 175

```
Ile Glu Glu Ala Leu Asp Ser Pro Lys Ser Tyr Asn Trp Ala Val Thr
        180                     185                     190

Lys Glu Gly Gln Val Val Arg Asn
    195                     200
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2852 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Rat 5'OT-EST-xdel

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1026..1270

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1799..2235

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1030..1152

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
TGACCTCTGT GGATCTGATA TACATGTAAG TGACAGACCA TCCGAGCTAT ATAGTGAGAC      60

CTGTGCAAGG AAGGATGGAG TGCACGTTCC CTGATGTTCA GAGCAACCCT GTGTCACTCC     120

AGGTAGGTGA GATGAGAGGA AGAGGGTGGC CTTGGCCTGG GCCTCCTACG GGCCTGGAAG     180

TTGGGAGAAG GATGTAAGCA GACTCTGTTC TCTTCTGAGA AATATCAGGT ATTGCAGTCA     240

GCCCAGGCTC CTCAGACCCT CCTAAGTGCA GATTCTCTGC AGAATCTGGT GTTGACAACA     300

CTAATGAGTA GGATGAGACT TCAGTTCCCT AGCCCTCACC GTCAGCTTCT GATTACCAAC     360

AACTCTCCCA GAGGAGAGCC ATCTACCTTT GGGACAGATG CTCTCTGCCC TGCACTGCCT     420

CCTGTTTCTC TTCATTGTAG AGGAAGATAG TACTTTAAAA GCTTCATAAA TGGTCTCAAG     480

GTGGGAAGAC CCCGGCTCAG GTGAAAGAGG ACAAGCGTCA CCTCACACAG GCCACCCAGT     540

AGAAAACAAG TGATCACTGA TACTGAGAAC TCTGGCAATT GCAGAGCTGC CCAAGACCAC     600

AACAGGGCAG TGCAATGCAA GGAAAAGGTT TGTTGCTCGA TTGCAAACCT AAAGTTTAAA     660
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GTGCATCAGG AGAACGCTTA CTCAAAGAGG AAGTGTAAGC CTAACTTAAG TAGCTAGAAG     720

CTCAGAATTT CTTGCATCAG CCCTGGAAGG GTACACAGGC CACCGGTGGG CCAGAGAACC     780

ACACGCTTTG GGGCGGTGTC CAAGCTTGTG AACAAGTAGG CAAGAGCGCC TGGTGTTGTA     840

GCTGTCATTG GCGGGCAATA CAGCCCAGCG AACTGTGGTC TCCAAGGTGC CCCTCGACCC     900

TCCCACTCTA CCCGAGACTC CAGGGACGCG ATGGGCCAGA CAGCAAGAGC TCCGCCTACG     960

GGGGCGGGGA CAGGAGATTC CCGTGATGCT CCTCGACCAC TTCCGGACAG GGCGCAGGCG    1020

CTAGCTGTC ATG TTG CGG GCT TTG AAC CGC CTG GCC GCG CGG CCC GGG        1068
          Met Leu Arg Ala Leu Asn Arg Leu Ala Ala Arg Pro Gly
                      205                     210

GGC CAG CCC CCA ACC CTG CTC CTT CTG CCC GTG CGC GGC CCA CGG CCC      1116
Gly Gln Pro Pro Thr Leu Leu Leu Leu Pro Val Arg Gly Pro Arg Pro
    215                 220                 225

CGC TCA TTC TCG GCT CCT TTT TCC TCG CAG GAT AGC TAGGTTGCAG           1162
Arg Ser Phe Ser Ala Pro Phe Ser Ser Gln Asp Ser
230                 235                 240

CTCGAAGCAC AGGCCCAGGA GCTGCGGCAG GCTGAGGTCC AGGCCCAGAG GGCCCAGGAG    1222

GAGCAGGCTT GGGTGCAACT GAAAGAACAA GAAGTTCTCA AACTGCAGGT GGGCCGAGGT    1282

CGTGAGGAAT GTGGGTATTG GAGATTCCGG TGAGGGAGGC TCTGGGGAGA GCAGCACAGG    1342

GTGTCAAGTG ACCAGTCTTC AGGAGGCTTC TCTCTCTGCT CTGCACACAC AGAGTGCCTC    1402

CCAGACAATG GTCAATGAAA GGTTACAGGC TAGTATTGCC GTGTGAAACT TGAAGGTCAG    1462

GGAAACCATA AATGAGAATG GAGCTGTTTT TATTGTGTAA GGGAGAGTGA CAAGGTTGAG    1522

AGAGTCCACC ACCCCGCACC TCCCCCCGCC CCCAATCAGG TTGTCACGAT TCGATTCGTT    1582

CTTGGGTTGT GGCTGAGAGA TCTGATGGGT AATTGTCCGA GGAAGAGGGA TATAATGGTT    1642

GAGGTCACCT AGTACAGTTG TGCTGGCCTA TTGGTGGGAC ACTCAAAGGG GCCCTGGGCT    1702

CTTTTGACAC CCTTCTTAAG GTGGGCTAGA GACAGTAAGT TATGCAGGCA GCCAGCTCTG    1762

AGAGATCCCA CGTAGCTAAC CTTTCTCTTC CCGTAGGAGG AGGCCAAAAA CTTCATCACT    1822

CGGGAGAACC TGGAGGCACG GATAGAAGAG GCCTTGGACT CTCCGAAGAG TTATAACTGG    1882

GCGGTCACCA AAGAAGGGCA GGTGGTCAGG AACTGAGAAC AGAGGCCTCT CAGGCCCAAA    1942

TAAGGACAGT GCTTGCCTAG GGACTGGATA TTGGGGTAGA AATTGGTGCA TCCCAGGAGG    2002

GTGGCACAGC CTTGTCCAGA GCAGCCCCCA TTCATTCTAG ATTTGGCACC AGGTATAGTA    2062

CCTGTTCTGA CACCACATAC AAACTCCGGA CAGCATTAAA CTCTGGGAAG TTCCTATCAC    2122

ACAGAAGATC AGACTGGACT GTCCCCTCTA GAAGCCAAGA GCTGTCTCCT GAGTTTCTTG    2182

GAATAGTGTG AGCCCAATGT TTCCTGCTTT TATAAATAAA CTATTGGAAA GCAAAGCCTT    2242

TTGTTATGTG GCTTGCTTTT TCTTGTTGTA GAATAAGTTT ATTTGTCCCA GTTATTTGGG    2302
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TCTTAAGGTT ATTAGCCAAA AGCCAGTTCA CCTAACTGAG CCAGGAGTTA GTTATCTGCT 2362

TTGCTCAATC CTGGGCTTTG CTGGGTAGGG TCAGGTGTGT CCAAGGTCCA GAAAGCAAAA 2422

AGGGTGCCCC GTTTCTCCTG GGAAGGCTTC CCCGTCAGTG ATTTCTGTAA CCGGACCCTG 2482

CCCTGACACA GCGTCATTGG ACTACCCAGC AGACAGTAGA CTCCACTCTA AACCCGCTTC 2542

TTGCGGTCAG TTGCTGTCCT TCAGTGTGTG TAAGCAGTGG CCAGACAGCA CCCTTGGGTG 2602

TCATTTCAAG ACTCTCTCAC CTTGGTCTGC TTTACGTTTG GTTTGATTTG GTTTGTTCTG 2662

GTTTTTGAGA CGAGGCCTTT CACTGGAACC TGGCACTCAG TATTTAGACT GCCCAGCCAG 2722

CTAGCCTCAG AGAATGCATC TGCGTATGCT TGCCTGGCGC TGGAATTCGG TGCACATGGC 2782

TTTGATGTGT ACCGGGGATC AGACACAGAT GTTTCATGAG TGCAGTGCAT GCCTGTTAGT 2842

GGTAGAGCTC 2852

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Leu Arg Ala Leu Asn Arg Leu Ala Ala Arg Pro Gly Gly Gln Pro
1 5 10 15

Pro Thr Leu Leu Leu Leu Pro Val Arg Gly Pro Arg Pro Arg Ser Phe
20 25 30

Ser Ala Pro Phe Ser Ser Gln Asp Ser
35 40

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTCACACCAC TCTGTCGAAC 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGGAGGAAGA CAGGTGAAAG 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCATGTTGCG GGCTTTGAAC 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TCTTTCAGTT GCACCCAAGC 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTGATAGGAA CTTCCCAGAG 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCCTCGTGCA ATTTCCCTCG CACCTCCAAT GTGAACTCTC GC 42

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TCCTGCGAGG AAAAAGGAGC CGAGAATGAG CGGGGCCGTG GG 42

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3264 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Rat 5'OT-EST

(ix) FEATURE:
(A) NAME/KEY: exon w
(B) LOCATION:1026..1241

(ix) FEATURE:
(A) NAME/KEY: exon x
(B) LOCATION:1332..1478

(ix) FEATURE:
(A) NAME/KEY: exon y
(B) LOCATION:1559..1682

(ix) FEATURE:
(A) NAME/KEY: exon z
(B) LOCATION:2211..2647

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TGACCTCTGT GGATCTGATA TACATGTAAG TGACAGACCA TCCGAGCTAT ATAGTGAGAC 60

CTGTGCAAGG AAGGATGGAG TGCACGTTCC CTGATGTTCA GAGCAACCCT GTGTCACTCC 120

AGGTAGGTGA GATGAGAGGA AGAGGGTGGC CTTGGCCTGG GCCTCCTACG GGCCTGGAAG 180

TTGGGAGAAG GATGTAAGCA GACTCTGTTC TCTTCTGAGA AATATCAGGT ATTGCAGTCA 240

GCCCAGGCTC CTCAGACCCT CCTAAGTGCA GATTCTCTGC AGAATCTGGT GTTGACAACA 300

CTAATGAGTA GGATGAGACT TCAGTTCCCT AGCCCTCACC GTCAGCTTCT GATTACCAAC 360

AACTCTCCCA GAGGAGAGCC ATCTACCTTT GGGACAGATG CTCTCTGCCC TGCACTGCCT 420

CCTGTTTCTC TTCATTGTAG AGGAAGATAG TACTTTAAAA GCTTCATAAA TGGTCTCAAG 480

GTGGGAAGAC CCCGGCTCAG GTGAAAGAGG ACAAGCGTCA CCTCACACAG GCCACCCAGT 540

AGAAAACAAG TGATCACTGA TACTGAGAAC TCTGGCAATT GCAGAGCTGC CCAAGACCAC 600

AACAGGGCAG TGCAATGCAA GGAAAAGGTT TGTTGCTCGA TTGCAAACCT AAAGTTTAAA 660

GTGCATCAGG AGAACGCTTA CTCAAAGAGG AAGTGTAAGC CTAACTTAAG TAGCTAGAAG 720

CTCAGAATTT CTTGCATCAG CCCTGGAAGG GTACACAGGC CACCGGTGGG CCAGAGAACC 780

ACACGCTTTG GGGCGGTGTC CAAGCTTGTG AACAAGTAGG CAAGAGCGCC TGGTGTTGTA 840

GCTGTCATTG GCGGGCAATA CAGCCCAGCG AACTGTGGTC TCCAAGGTGC CCCTCGACCC 900

TCCCACTCTA CCCGAGACTC CAGGGACGCG ATGGGCCAGA CAGCAAGAGC TCCGCCTACG 960

GGGGCGGGGA CAGGAGATTC CCGTGATGCT CCTCGACCAC TTCCGGACAG GGCGCAGGCG 1020

CTAGCTGTCA TGTTGCGGGC TTTGAACCGC CTGGCCGCGC GGCCCGGGGG CCAGCCCCCA 1080

ACCCTGCTCC TTCTGCCCGT GCGCGGCCGC AAGACCCGCC ACGATCCGCC TGCCAAGTCC 1140

AAGGTCGGGC GCGTGAAAAT GCCTCCTGCA GTGGACCCTG CGGAATTGTT CGTGTTGACC 1200

GAGCGCTACC GACAGTACCG GGAGACGGTG CGCGCTCTCA GGTGTGTGTA AAGGGCAGGC 1260

GGCCTTCGGC GCCCCCTGGG AAGTGCTGGG GCTGGAGGAT GGGTGCTCAC TTGAAGCCCG 1320

TCCTCACCCA GGCGAGAGTT CACATTGGAG GTGCGAGGGA AATTGCACGA GGCCCGAGCC 1380

GGGGTTCTGG CTGAGCGCAA GGCGCAAGAG GCCATCAGAG AGCACCAGGA GCTGATGGCC 1440

TGGAACCGGG AGGAGAACCG GAGACTGCAG GAACTACGGT GCGAGAGGCG CGGGGCTGGG 1500

TGGGCTGGGC TAGGCTCACC CACGGCCCCG CTCATTCTCG GCTCCTTTTT CCTCGCAGGA 1560

TAGCTAGGTT GCAGCTCGAA GCACAGGCCC AGGAGCTGCG GCAGGCTGAG GTCCAGGCCC 1620

AGAGGGCCCA GGAGGAGCAG GCTTGGGTGC AACTGAAAGA ACAAGAAGTT CTCAAACTGC 1680

AGGTGGGCCG AGGTCGTGAG GAATGTGGGT ATTGGAGATT CCGGTGAGGG AGGCTCTGGG 1740

GAGAGCAGCA CAGGGTGTCA AGTGACCAGT CTTCAGGAGG CTTCTCTCTC TGCTCTGCAC 1800

ACACAGAGTG CCTCCCAGAC AATGGTCAAT GAAAGGTTAC AGGCTAGTAT TGCCGTGTGA 1860

AACTTGAAGG TCAGGGAAAC CATAAATGAG AATGGAGCTG TTTTTATTGT GTAAGGGAGA 1920

GTGACAAGGT TGAGAGAGTC CACCACCCCG CACCTCCCCC CGCCCCCAAT CAGGTTGTCA 1980

CGATTCGATT CGTTCTTGGG TTGTGGCTGA GAGATCTGAT GGGTAATTGT CCGAGGAAGA 2040

GGGATATAAT GGTTGAGGTC ACCTAGTACA GTTGTGCTGG CCTATTGGTG GGACACTCAA 2100

AGGGGCCCTG GGCTCTTTTG ACACCCTTCT TAAGGTGGGC TAGAGACAGT AAGTTATGCA 2160

GGCAGCCAGC TCTGAGAGAT CCCACGTAGC TAACCTTTCT CTTCCCGTAG GAGGAGGCCA 2220

AAAACTTCAT CACTCGGGAG AACCTGGAGG CACGGATAGA AGAGGCCTTG GACTCTCCGA 2280

AGAGTTATAA CTGGGCGGTC ACCAAAGAAG GGCAGGTGGT CAGGAACTGA GAACAGAGGC 2340

CTCTCAGGCC CAAATAAGGA CAGTGCTTGC CTAGGGACTG GATATTGGGG TAGAAATTGG 2400

TGCATCCCAG GAGGGTGGCA CAGCCTTGTC CAGAGCAGCC CCCATTCATT CTAGATTTGG 2460

CACCAGGTAT AGTACCTGTT CTGACACCAC ATACAAACTC CGGACAGCAT TAAACTCTGG 2520

GAAGTTCCTA TCACACAGAA GATCAGACTG GACTGTCCCC TCTAGAAGCC AAGAGCTGTC 2580

TCCTGAGTTT CTTGGAATAG TGTGAGCCCA ATGTTTCCTG CTTTTATAAA TAAACTATTG 2640

GAAAGCAAAG CCTTTTGTTA TGTGGCTTGC TTTTTCTTGT TGTAGAATAA GTTTATTTGT 2700

CCCAGTTATT TGGGTCTTAA GGTTATTAGC CAAAAGCCAG TTCACCTAAC TGAGCCAGGA 2760

GTTAGTTATC TGCTTTGCTC AATCCTGGGC TTTGCTGGGT AGGGTCAGGT GTGTCCAAGG 2820

TCCAGAAAGC AAAAAGGGTG CCCCGTTTCT CCTGGGAAGG CTTCCCCGTC AGTGATTTCT 2880

GTAACCGGAC CCTGCCCTGA CACAGCGTCA TTGGACTACC CAGCAGACAG TAGACTCCAC 2940

TCTAAACCCG CTTCTTGCGG TCAGTTGCTG TCCTTCAGTG TGTGTAAGCA GTGGCCAGAC 3000

AGCACCCTTG GGTGTCATTT CAAGACTCTC TCACCTTGGT CTGCTTTACG TTTGGTTTGA 3060

TTTGGTTTGT TCTGGTTTTT GAGACGAGGC CTTTCACTGG AACCTGGCAC TCAGTATTTA 3120

GACTGCCCAG CCAGCTAGCC TCAGAGAATG CATCTGCGTA TGCTTGCCTG GCGCTGGAAT 3180

TCGGTGCACA TGGCTTTGAT GTGTACCGGG GATCAGACAC AGATGTTTCA TGAGTGCAGT 3240

GCATGCCTGT TAGTGGTAGA GCTC 3264

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44576 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Cosmid DNA"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCGGCCGCAT AATACGACTC ACTATAGGGA TCTGGTGGAG GACCTATGGC CCGCGAGCTA 60

GAGAAGTGGT TCTCAACCTT CCTAGTGCTG AGACCCTTTA ACACAGTTCC TCGTGTTGTG 120

GGGAAACCCC CTCCTGCAAC CATAAAATAA TTTTTGTTAC TACTTCATAA CAAGTGTTGC 180

TACTCTATTG CTATGAATTG TAAAATAAAT GTGTCTTCCA ATGGTCTTAG ATGACTCCCG 240

TGAAAGGGTC ATTCTACCCC TAAGAGGTCA TGATCTACAG GTTGAGAACC ACTGATCTCC 300

AGTAACCTTC ACTTGAGTCC ATATCCTCCA TGAAGGTATG GAAGTCAATA AAACTGAGCT 360

TCAAGCCTCA TCAAAATGGG TCCATCCCCT GGTACAGTGT GAGTGGAAGA ATACCCACCA 420

TACGGTCACT GGAAGGAGGA TGTCTGAAGG GTCTTAGATT GTGTCAAGGG GTCCTGGGTG 480

TCAGGATCTG ACGAAGCAGG CTCGTCATGT TTCATGAAGA CTACAGGTAT GTGATAAAAC 540

TGCAAGCTGG AAAAGTACCC ACTGAGCCCG TGTGGCTCTG CTGGGATTTG GAGGCATGAG 600

GAGCAGAGGG TCTGGAGGAC AGCAGTCCCA GAAATAATCT ATGACTAAGA AGGCTGAACT 660

GGGGTGACTC TCTGGTGGAA AGAGTTGCCT TTTAAGAAGG AAGACATACC AGGCATAGCA 720

ACAACTGCCT TTAGTACTAG CACTCTGAAG GCAGAGGAAG TCCGATTTCT CTGAGTTCCA 780

AGCCAGCTTG GTTTACACAG CAAGTTCTAG GCCAACTAGG GTTACATAGT GGACTCTCCT 840

CAAACGGGGT TGAGAAAGGA CTCAGCAGTT AGCTCAGTTA ACTCCAGTTC TAGGAAATAT 900

GATCCCTTAG TCTGACCTCT TGGCATGTAA GTGGTGCACA TACATATATG CACACAAAAT 960

ACATCAATCT GCAAAGGGGG AGGGAGGAAG GGCTGGAGTC TGAAGAAATA GTTCAGTGGT 1020

TAAGAGAATT CACTGCTCTT CCCAATAGCC AAATTCAGCT CCTAGCATCC ATGTCAGATG 1080

GCCCACGAAC ACCTGTAATT CTAGCCCCTA AACTCAGTGC CCCTTCACAA GACGGGGACA 1140

CACGTACACA TATACCTAAA AAATTAGGTG GTTTTTTTTT ATTTATAAGG TCAAATGCAG 1200

AATATCAAAT GGGTTAGACA GCAGCTCCAA GCTGGCCTCT TCCTCCCAGG GCTCTTCTTG 1260

ACTCTTGGCA CCCTCTTTGG GTCCAGAACC CAGACATTAG CCATGACTCA GCTGATAAAA 1320

TGCAACCCAT GGCTCATTAA TTAGGAAGTC TGTAATTAGC CTGTCTGGTA GCCTCCAGAG 1380

AGAACCCCTT TCACCTGTCT TCCTCCTCTC ACCCAGGGGA AGAGCTCAGT TTTGCCCCTG 1440

AGACAGAAGA AGGGAACGAG ACCATGAGCA ACGGGAAATG AGATGCTGGC GCACACACAC 1500

TTTATGTGTG TGAAGTCTCA GAGAGGTCAC CAATAATGAG GCAATGGAAA TGAGCTGAGC 1560

TGCCTGAACC TCCAAGTTTC CTCCAAGAAA ACCCCACAGG GGAGATGGGG CATGGCCCAG 1620

GCCAGCTGCC CCAGCCTCTG CTGGCAGAAA GTGAGCCCGC TGCCATTTTA ATTTTTGATA 1680

CAGGGTCTCA CTCTACAGCT CTGGGGGCCT AAAACTCACT ATGTAGACTT CAAACTCAAC 1740

CAAACCAACA ACAAAAACAA ACAAAACCCC TGCACTGACT GGAGAGATGG CTCGGTTGAG 1800

AACAATGGCT GCTAGGAGTC AAACCCAGGT CCTGTGGAAG AGCATGCTGG TAACTGCTGG 1860

GTCATCGCTG	GGTCACTCTC	TTCACACACA	CACACACACA	CACACACACA	CACGGCAATG	1920
AACTCTTCAG	TGTCTTGATT	TACGGTTTCT	TCCGATAAAT	CCTCAGGAGG	GCAGTCAAGT	1980
GGCTCATTTG	GCAAATGCTT	GCCTGAGACC	TGAGTTTGGT	TCCCAGAACC	CATGGAGGCA	2040
GAAGGAAAGG	GCTCCACAAA	GCTCTCTTCT	GAACTCCATA	TGTGCACACA	CACCCACTTC	2100
GCACACATTC	ATAATAGTGA	TGAATGAAAA	TGAAGACAGA	TAAAAAAAAC	CAATTTCGTG	2160
AAACTGTTAG	CACGTTCAGT	CAATGGCTTT	GGGGGTAACC	TGTTTCAGAG	CCATGGTACT	2220
CAGTCACTAG	GCTCATACTG	GTCAGACGCT	GAGGTCAGCA	ATGGAGAGCT	GCTACACCTA	2280
AAGGTAGCAG	AGGTCATTTG	GCTCTGACTC	AGAATATTCC	AGCTCTCCAC	ATTCACAGAA	2340
GTTCTACTTG	GTCGTAGAAA	AAAGCTGAGC	CTTTTTTTTT	TTTTGGAACT	TTATTTTTTT	2400
AAAGATATAT	TTATTTTATG	TATATGAGTG	CACTGTAGCT	GTCTTCAGAC	ACACCAGAAG	2460
GGGGCATCGG	ATCCCATTAC	AGATGGCTGT	GAGCCAACAT	GTGGTCGCTG	GGGATTGAAC	2520
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CCTTCAGTCA GCTGAGCAGT TTGCCCTCGC TCGGCTGCAG TACCAGCACA GGCACCCCAG 32940

TCTCCCCGAT GAGCGGTCTC ATGAGGATTC AGGTTGGTCT CGAACTCCCT ATGTAGCTGA 33000

AAATGACCTT GAGATTCTAC CAATCCTCTT GCCTCTTCCT CTAAAAGCAG GCAGTGAACC 33060
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(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGACAGCCCG AAGGACTACA GGT 23

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CGAAGAACTC CGCAGGGTCC 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAGACCCGCC ACGACCCG 18

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GAATCAGCAC CCTCTCCGCC 20

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TGCGGAGTTC TTCGTGCTGA TGGAG 25

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGTGCTCGGC GGCGTCCTTC 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GAGTGGCGGA GAGGGTGCTG A 21

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GGCCGAGGCT GAGCGGGG 18

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CTGAAGGACG CCGCCGAGCA 20

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CTCCAACGCC TGCCGCTGC 19

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCAGGAGGAG CGGGAGCAGG A 21

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic Primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCCAGTGCCC CGCAAGCCG 19

International Application No
PCT/GB 99/02658

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N5/10 C07K14/47 A01K67/027 G01N33/50
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL SEQUENCES 'Online! Accession No. AI011995, 17 June 1998 (1998-06-17) LEE N.H.: "EST; rat cDNA clone RPLAR4" XP002127678 corresponds to nt 86-614 if seq. ID 1	9
P,X	DATABASE EMBL SEQUENCES 'Online! Accession No. AI180224, 12 October 1998 (1998-10-12) LEE N.H.O: "EST; rat cDNA clone EST223967" XP002127679 complementary to nt 1798-2234 of seq. ID 4	9,10

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 January 2000	28/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	MOHR E. ET AL.: "A single rat genomic DNA fragment encodes both the oxytocin and vasopresson genes separated by 11 kilobases and oriented in opposite transcriptional directions." BIOCHIMIE, vol. 70, no. 5, May 1988 (1988-05), pages 649-654, XP000867203 cited in the application the whole document	1-29
A	HO M.-Y. ET AL.: "Bovine oxytocin transgenes in mice" J. BIOL. CHEM., vol. 270, no. 45, 10 November 1995 (1995-11-10), pages 27199-27205, XP002127677 the whole document	1-29
A	WALLER S.J. ET AL.: "Transgenic and transcriptional studies on neurosecretory cell gene expression." CELL. MOL. NEUROBIOL., vol. 18, no. 2, April 1998 (1998-04), pages 149-171, XP000867082 the whole document	1-29
A	KLEBIG M L ET AL: "ECTOPIC EXPRESSION OF THE AGOUTI GENE IN TRANSGENIC MICE CAUSES OBESITY, FEATURES OF TYPE II DIABETES, AND YELLOW FUR" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 92, May 1995 (1995-05), page 4728-4732 XP002037980 ISSN: 0027-8424 cited in the application the whole document	1-29
A	US 5 789 651 A (WOYCHIK RICHARD P) 4 August 1998 (1998-08-04) abstract	1-29
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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